Preparation of Gelatin Hydrogels with Different Stiffness and Pore Structures and Their Application for Cartilage Tissue Engineering

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List of abbreviations

ANOVA	Analysis of variance
bFGF	basic fibroblast growth factor
BMSC	Bone mesenchymal stem cell
GelMA	Gelatin methacrylate
GelMAGMA	Glycidyl methacrylate GelMA
CLSM	Confocal laser scanning microscopy
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
NMR	Nuclear Magnetic Resonance
AFM	Atomic Force Microscope
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
HA	Hyaluronic acid
HE	Hematoxylin and eosin
CS	Chondroitin sulfate
IGF	Insulin growth factor
MW	Molecular weight
PBS	Phosphate buffer saline
PVA	Poly(vinyl alcohol)
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PGA	Poly(glycolic acid)
RGD	Arginylglycylaspartic acid
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
PEG	Poly(ethylene glycol)
PEGDA	Poly(ethylene glycol) diacrylate
TGF-β	Transforming growth factor β
sGAG	Sulfated glycosaminoglycan
HAMA	Methacrylated hyaluronic acid
UV	Ultraviolet
EO	Ethylene oxide
3D	Three-dimensional

Chapter 1

General introduction

1.1 Cartilage tissue engineering

1.1.1 Articular cartilage and defect

Articular cartilage presents like a white layer on the surface of long bone ends, is the most important tissue for movement. Because it can withstand weight-bearing and work as a lubricant to decrease the friction between the joints. The cartilage is comprised of dense extracellular matrices while almost no vascularity. The extracellular matrix (ECM) of articular cartilage is composed of aggrecan and collagen type II, which offer strong mechanical property and smooth surface after assembly into a complex three-dimensional (3D) network structure. As shown in Figure 1.1, aggrecan contains core proteins and link proteins with abundant glycosaminoglycan (GAG) chains. Due to the heavy pressure or injury during strenuous exercise, the cartilage is easily damaged. The defect will lead to inflammation and degeneration of the cartilage which induce severe joint pain and unfree mobility. Unfortunately, the self-healing capacity of cartilage is very limited due to its lack of vasculature for nutrition supply.[1]

1.1.2 Traditional treatments and their limitations

Conservative measures are helpful for the early phase of chronic cartilage defects, such as using the anti-inflammatory drug that can calm the inflammatory response.[2] But this kind of the drug may have side effects. The purpose of cartilage repair is to restore the healthy joint surface with weight bearing capacity and without pain. Surgical treatments are needed if the defect is serious, which include drilling, abrasion arthroplasty and microfracture.[3-5] Among these treatments, microfracture is one frequently used method. The aim of this surgery is to induce the blood and stem cells from bone marrow to the defected area by puncturing the subchondral.[6,7] However, the newly formed cartilage using this method is fibrocartilage rather than hyaline cartilage. From composition point, the fibrocartilage contains much less proteoglycan and more collagen type I instead of collagen type II than native hyaline cartilage. Therefore, in this fibrocartilage

area, the ability of compression withstanding and offering smooth surface decreases over time. Furthermore, the repaired cartilage shows bad integration with residual cartilage.

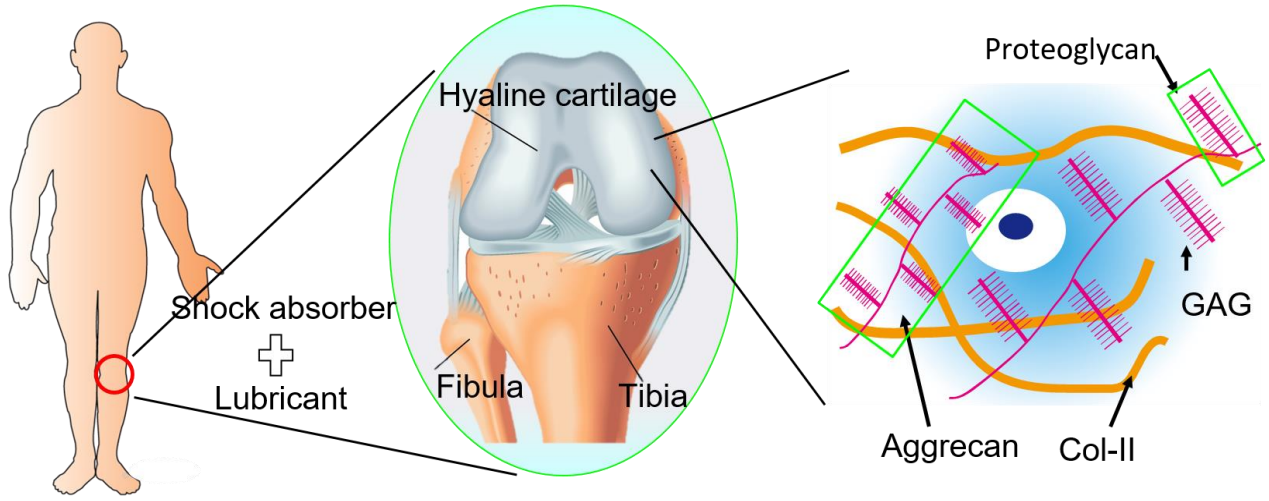

Figure 1.1 Structure and ECM of articular cartilage

1.1.3 The principles of cartilage tissue engineering

To address the challenge of cartilage regeneration, an approach is highly needed to replace the traditional treatments. Cartilage tissue engineering has been developed as a promising approach to solve the problems of traditional treatments. Cartilage tissue engineering combines cells, scaffolds and bioactive molecules to induce the repair and regeneration of cartilage tissue (Figure 1.2). During this process, the critical part is scaffold that has the function to create a good microenvironment for cell activities, such as cell adhesion, cell proliferation, secretion and assembly of the ECM.

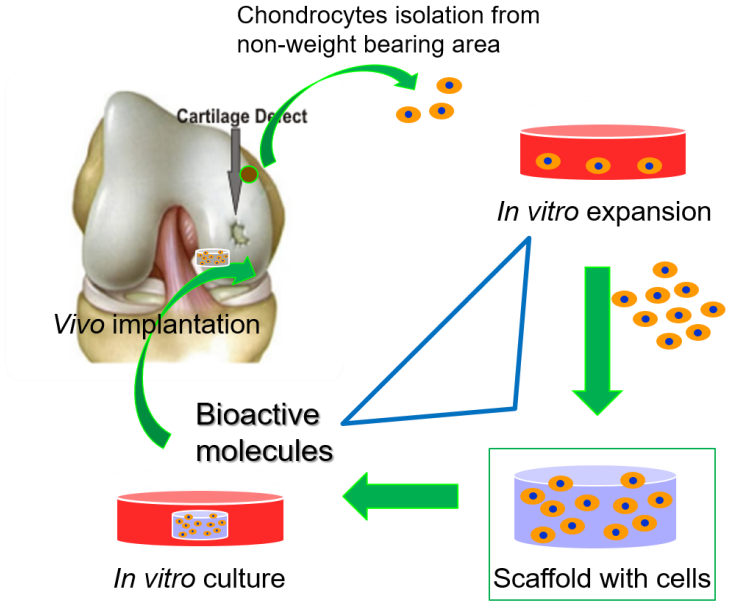

Figure 1.2 Principles of cartilage tissue engineering

1.1.4 Cells

Healthy chondrocytes can be arthroscopically isolated from the non-weight bearing area of the cartilage.[8] Chondrocytes obtained through this method is the best source, but usually, the cell number is very limited.[9] Chondrocytes can proliferate to a large cell number during *in vitro* expansion to meet the requirements of tissue engineering. However, during the *in vitro* expansion, chondrocytes are easy to lose the chondrogenic phenotype.

Besides the mature chondrocytes, adult stem cells have been studied a lot for cartilage regeneration. The adult stem cells can proliferate and keep the potential for differentiation into other specialized cells. One of such kinds of adult stem cells is mesenchymal stem cells, which can be derived from many tissues, such as bone marrow, adipose tissue and synovial tissue.[10-12] In the situation of shortage of chondrocytes, these stem cells can be used for cartilage tissue regeneration after chondrogenic differentiation.

1.1.5 Bioactive molecules

To promote the cell functions and facilitate cartilage tissue regeneration, bioactive molecules, such as growth factors are frequently used. In tissue engineering, growth factors are commonly used to promote cell proliferation and differentiation. There are several common growth factors for cartilage tissue engineering, such as basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β). The former one can promote cell proliferation while keep the cell phenotype. TGF-β shows good function for maintenance or redifferentiation of chondrocyte phenotype.[13] Furthermore, it also exhibits very effective effects on chondrogenic differentiation of stem cells.[14]

1.1.6 Scaffolds

In cartilage tissue engineering, scaffolds serve as temporary templates to accommodate cells and provide various signals for cell adhesion, proliferation, differentiation and ECM secretion. After all the processes, the scaffolds should be degraded to further provide spaces for the final tissue regeneration. During the maturation of cell/scaffold construct, the scaffolds should provide suitable biomechanical and biochemical stimuli for the cell activities. Based on the principle of tissue engineering and functions of the scaffolds, the following requirements are summarized for scaffold designs.

I. Biocompatibility

This character is the most important property of scaffolds. Compared with artificial prosthesis replacements, the advantage of tissue engineering is bioactivity due to the cell-laden characteristic. Therefore, materials and methods for scaffolds fabrication should be biocompatible to allow cell attachment, proliferation and differentiation.[15] The safety of scaffold degradation products should also be considered. The aim of tissue engineering is the application in clinic. The effects of scaffolds on the surrounding tissues and inflammatory responses should also be taken into account to make sure the biocompatibility of scaffolds.[16]

II. Biodegradability

The scaffolds-forming materials should be metabolically degradable by the body.[17] The scaffold

degradation speed is very important. Quick degradation will affect the integrity of scaffolds and further influence the tissue regeneration. But if the degradation is too slow, it will inhibit the new tissue formation. Therefore, the proper speed of hydrolytic or enzymatic degradation is necessary for cartilage tissue regeneration.[18]

III. Biomechanical property

Biomechanical property of scaffolds should be designed from two aspects. One is easy operation and withstanding deformation. The other is scaffolds should be strong enough to be handled and be stable during *in vitro* culture and *in vivo* implantation.[19] Especially for cartilage tissue engineering, high mechanical property is needed for the weight bearing. Another requirement is the proper mechanical stimulus for the encapsulated cells. Because many studies have shown that mechanical stimulus can affect the cell fates.[20]

IV. Biochemical property

The materials composition and chemical properties are important for cell functions and engineering a good 3D tissue. For example, many studies have reported that the surface charge of the matrices can affect the differentiation of stem cells.[21] Modification of scaffolds with arginylglycylaspartic acid (RGD) motifs has been reported to improve cell viability and activity.[22]

V. Sterilizability

Scaffolds should be sterilized by a simple way without loss of functions. The sterilization methods include filtration (0.22 μm filter), heating, ethylene oxide (EO) gas and ethanol immersing. The methods and protocols should be chose depending on the property of scaffolds.[23]

1.2 Scaffolds for cartilage tissue engineering

1.2.1 Materials for scaffold fabrication

a) Synthetic materials

Synthetic materials, such as PEG, PLGA and PVA are usually used for scaffold fabrication. The advantages of these materials are the reproducibility and controllability, which are the result of specific molecular weights (MW) and block structures (Figure 1.3).[24] However, the drawback of synthetic materials is the limited biological properties, which control the cells and matrices interaction.

I. Poly(ethylene glycol) PEG

PEG has been approved by FDA for several biomedical applications due to the low toxicity. PEG hydrogels have been widely studied as cell-laden scaffolds and drug carriers. It can be modified with acrylate or methacrylate to form photocrosslinkable hydrogels.[25] The block copolymer of PEG and PLLA can form a thermally reversible hydrogel.[26] PEG hydrogels with high mechanical property have been used to encapsulate chondrocytes for cartilage tissue engineering.[27] However, the lack of bioactivity and biodegradability of PEG limit the application for long time 3D cell culture.

II. Poly(vinyl alcohol) (PVA)

PVA has been used for many biomedical applications due to the good biocompatibility. Physically

crosslinked PVA hydrogels can be formed by several freezing and thawing cycles. However, the critical disadvantage of PVA is nonsupport of cell adhesion, which causes the seldom usage for 3D cell culture.[28]

III. Poly(lactic acid-co-glycolic acid) (PLGA)

PLGA is the copolymer of glycolic acid and lactic acid. PLGA has been frequently used for pre-made porous scaffold preparation as it has good biodegradability, biocompatibility and excellent mechanical property. The biodegradability can be controlled by the ratio of glycolic acid and lactic acid.[29] However, PLGA is difficult to be dissolved in aqueous solution, which limits the usage for hydrogel fabrication.

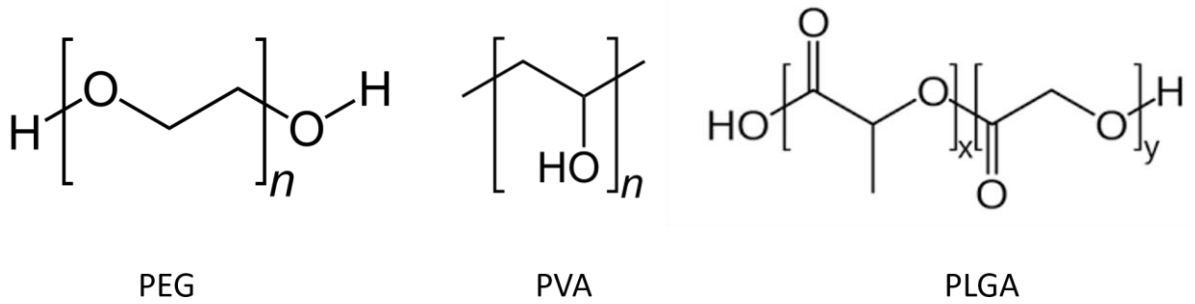

Figure 1.3 The chemical structure of PEG, PVA and PLGA.

b) Natural materials

Natural materials have drawn more attention in tissue engineering compared with synthetic materials because of their good biocompatibility and bioactivity.[30] The sources of these biopolymers are extensive, such as humans, animals, plants and bacteria. The protein-based materials like collagen and fibrin exhibit good biocompatibility and enzyme-degradable property, which are the main components of mammalian tissue ECM.[31]. The other native polymers include hyaluronic acid, chondroitin sulfate, alginate and chitosan.[32-34] The structures, functions and applications of these natural materials are summarized below.

I. Hyaluronic acid (HA)

HA as a glycosaminoglycan is commonly prevalent in body liquid, such as the synovial fluid in joints and various tissue ECM. However, the commonly used HA is produced from bacteria rather than animals due to the productivity and cost.[35] Fermentation method can eliminate the possibility of disease transfer.[36] The biological functions of HA include lubrication, enhance the mechanical property of tissue and control the cell behaviors. The super high molecular weight of HA allows it to maintain the elastoviscosity of joint synovial and eye vitreous fluid. HA also plays a significant role in the assembly of proteoglycan in the cartilage ECM, which is important for the lubrication function of connecting tissue. Many of cell behaviors, such as migration, differentiation and inflammation are affected by the bonding between HA and cell surface receptors including CD44, ICAM-1 and RHAMM. It has been reported HA can bond chondrocytes through CD44 receptor and promote the redifferentiation of dedifferentiated chondrocytes.[37] HA has also been used for the wound healing. The wound contraction and re-epithelialization can be enhanced by more bonding of RHAMM with HA scaffolds.[38] The adhesion molecule (ICAM) is widely distributed on the surface of endothelial and macrophages. Chen et al. have reported that the ICAM-1 binding to HA-based scaffolds may control the inflammation activation.[39] The structure of HA is repeated disaccharides with β-D-glucuronic acid and N-acetyl-β-D-glucosamine units (Figure 1.4). Therefore, the functional groups (carboxyl and hydroxyl) can be used to crosslink with other bioactive molecules. For example, the

HA-collagen scaffolds have been prepared by using EDC/NHS crosslinking. The mechanical property and chondrogenic functions have been studied.[8,40] After inducing photoreactive groups by modification with glycidyl methacrylate, HA derivative can be crosslinked to form photocrosslinkable hydrogels for tissue engineering.[41]

II. Chondroitin sulfate (CS)

CS is sulfated glycosaminoglycan with a linear structure existing in cartilage tissue ECM. It is composed of disaccharide (N-acetylgalactosamine and glucuronic acid) and attached to proteins to form proteoglycan that is important for cartilage functions.[42] The location of sulfate groups classifies the CS as chondroitin-4-sulfate and chondroitin-6-sulfate. It is reported that CS in cartilage ECM provides the ability to resist the compression. It has also been shown that CS can promote GAG synthesis and chondrocyte proliferation.[43] ECM mimicking scaffolds can be prepared by adding CS. Chondrocytes cultured in such kind of scaffolds have round morphology, enhanced gene expression and secretion of cartilaginous ECM.[44] CS and HA are the main component of GAG in cartilage. Addition of HA/CS in scaffolds is useful for chondrogenesis.

III. Alginate

Alginate is an anionic biopolymer obtaining from bacteria and brown seaweed.[32,45] Alginate is comprised by (1–4)-linked β-$_D$-mannuronicacid (M) and α-$_L$-guluronic acid (G) monomers. The ratio of these monomers is depending on the various sources.[46] Because of its biocompatibility, low toxicity and cost, more importantly, the mild crosslinking method by divalent ions, alginate has been used for many biomedical applications including drug delivery and cell transplantation. For example, alginate hydrogel crosslinked by adding calcium sulfate can be used to encapsulate chondrocytes. The hydrogels have been reported to enhance secretion of chondrogenic ECM and increase of mechanical property.[47] The mechanism of ion crosslinking is the divalent cations, such as Ca^{2+} binding with G monomers. Therefore, the composition ratio and molecular weight are the main factors to affect the physical properties of alginate hydrogels.[48] Although alginate has good biocompatibility, it also has been reported that immunogenic response exists in high M content alginate and unpurified alginate.[49] This ionically crosslinked alginate scaffold undergoes uncontrolled dissolution due to the ion exchange *in vitro* and *in vivo*.[50] After dissolution, the molecular weight of alginate is still higher than the clearance threshold of the kidneys. There is no enzyme to degrade alginate *in vivo*. Therefore, it is likely the alginate cannot be removed from our body completely.[51]

IV. Chitosan

Chitosan is linear polysaccharides derived from chitin that is abundant in some plants or animals, such as crabs and shrimps. The deacetylation treatment can produce chitosan from chitin and change the crystallinity of the polymers. The stable and crystalline structure makes chitosan insoluble in physiological solutions. The free amino groups in chitosan are protonated in an acidic environment, which makes chitosan soluble.[33] The chemical structure is repeated (1–4)-linked $_D$-glucosamine and N-acetyl-$_D$-glucosamine. Because chitosan is structurally similar to GAG and can be degradable by the enzymes in the body, it has been studied for many kinds of tissue engineering applications. But the unsolvable property in neutral aqueous solution limits the applications for the 3D cell encapsulation.[33] Many studies have used the cationic nature of chitosan for biomedical applications. This charge density allows chitosan to form complex coacervates with anionic polymers, such as alginate and insoluble ionic complexes with ions, such as Fe.[52,53]

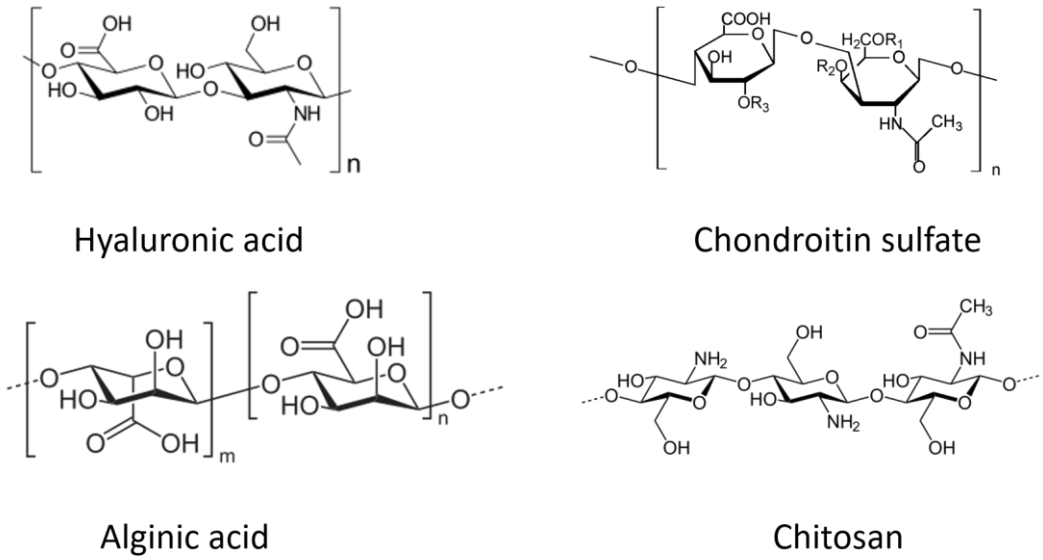

Figure 1.4 The chemical structure of HA, CS, Alginic acid and Chitosan. (CS: R1, R2, R3 = H or SO_3H.)

V. Collagen

Collagen is attractive for fabrication of scaffolds because it is the main ECM component of various tissues and the most abundant protein.[54] There are many types of collagen depending on the different spatial structure which is formed by three collagen chain rope together.[31] This helix structure of collagen can further assemble into fibrils by hydrogen bonding, which can be packed closely to form collagen fibers.[55] Compared with other biopolymers, collagen can be degraded by many kinds of enzymes including metalloproteases, collagenase and serine proteases.[56] This property allows the collagen scaffolds to be replaced with the secreted native ECM after long-term implantation. Collagen scaffolds have been developed with different structures and properties for biomedical applications. It has been reported that collagen scaffolds with interconnected porous structures can be fabricated by mixing with ice particles. These collagen scaffolds show excellent biocompatibility and functions for various kinds of tissue regeneration, such as skin, cartilage and bone.[57,58]

VI. Gelatin

Collagen has many advantages for tissue engineering. However, collagen contains some antigens which may induce immunogenicity when implanted *in vivo*.[59] In order to eliminate the collagen potential immunogenicity, gelatin as a substitute is widely used for scaffold fabrication. Except thermal treatment, gelatin can be derived from collagen by acidic (type A) or alkaline treatments (type B) to degrade the helix structures of collagen into the single chain by hydrolysis.[60] Luckily, after the treatment, gelatin shows almost no immunogenicity while still keeping many advantages of collagen. For example, gelatin polymer chain keeps the cell adhesion peptides and metalloproteinase degradable peptides. The tripeptide sequence RGD promotes the cell adhesion that is important for cell viability and proliferation.[61] The degradable sequence is related to the biodegradability of scaffolds and affects the cell-matrices interaction. The other attractive point of gelatin as biomaterial is the low cost and easy availability from many animals like pigs and cows. Gelatin can be easily modified and used to fabricate porous scaffolds and hydrogels for the regeneration of damaged tissues.[62] The previous results have demonstrated the good functions on the cultivation of different cell types and tissue regenerations.[63,64]

VII. Fibrin

Fibrin is a biopolymer consisted of fibrinogen. The fibrinogen molecule has two sets of three polypeptide chains that are bonded together by six disulfide bridges.[65] Fibrin monomer is formed after treatment by thrombin and has the tendency to assemble into insoluble fibrin. In vivo, the blood coagulation factor XIIIa works as a transglutaminase which crosslinks γ chains in the fibrin polymer by introducing intermolecular bonds between the lysine of γ chain and glutamine of the other.[66] After crosslinking, the fibrin network cannot be degraded by protease easily.[67] To fabricate tissue engineering scaffolds, fibrin network can be built by using chemical crosslinkers, such as genipin.[68] Fibrin hydrogel crosslinked by chemical crosslinker is not suitable for cell encapsulation due to the cytotoxicity. Fibrin hydrogel crosslinked by thrombin usually cannot be injected and has low mechanical properties.[69]

VIII. Silk fibroin

Silk fibroin is another protein based biopolymer for tissue engineering. It can be obtained by the treatment of silk fibers produced by silkworms and spiders.[70] The purpose of the treatment for the cocoon silk is to remove the sericin protein coated on the surface of silk fibroin core protein, which may cause immune responses. Silk fibroin exhibits strong mechanical property and good biocompatibility which make the silk fibroin as a suitable candidate for tissue engineering. Silk fibroin has also been developed as a potential matrix for drug releases, such as insulin-like growth factor I and surface coating.[71,72] Usually, the beta-sheet structure formation will make the fibroin insoluble, which works as physical crosslinking. For example, silk fibroin can be treated with methanol after freeze-drying to form porous structure and to obtain stable porous scaffold in aqueous solution.[73] Some treatments including vortex and sonication can induce silk fibroin aqueous solution to form hydrogels quickly.[74,75] However, these methods are not suitable for cell encapsulation because the crosslinking environments are unfriendly for the cells.[76]

1.2.2 Methods for scaffold fabrication

The functions of scaffolds depend on the materials properties and the structures of scaffolds. Scaffolds can be categorized into (a) porous sponges, (b) fabric scaffolds and (c) hydrogels according to the structures (Figure 1.5).

a) Porous scaffolds

The critical abilities of scaffolds are promoting cell adhesion and providing space for proliferation of the accommodated cells. There are many methods to form porous structures, such as gas forming, phase separation and leaching of porogen including sodium chloride, paraffin and ice particles.[77-81] Among the various porogen, the ice particles can induce formation of porous structures with homogeneous distribution and good interconnectivity. The pore structures can enhance the nutrient and metabolic waste exchange and promote tissue regeneration in the inner part of scaffolds.[58]

b) Fabric scaffolds

Fabric scaffolds possess high surface area and interconnected structures, which make them attractive for tissue engineering. The methods for fabrication of fabric scaffolds include electrospinning, self-assembly, phase separation and other techniques.[82-85] The limitation of this method is its low efficiency and difficulty to increase the thickness while keeping the good porous structures.

c) Hydrogels

Hydrogels are physically or chemically crosslinked polymer networks that absorb plenty of water and swell in aqueous solution. Hydrogels have many advantages because they can provide a three-dimensional microenvironment by encapsulation of transplanted cells in the hydrogel matrices and can be delivered to defects by low-invasive injection. In hydrogels, transplanted cells are encapsulated in the hydrogel matrices that can provide similar *in vivo* microenvironments for cell differentiation and maintenance of phenotype.[86-89] The obvious differences of hydrogels compared with porous sponges and fabric scaffolds are the sterilization and cell seeding method. The precursor solution of hydrogels can be sterilized by filtration before gelation. And due to the sol-gel translation, cells can be encapsulated in precursor solutions before injection and gelation. These advantages make hydrogel more attractive for cartilage tissue engineering.

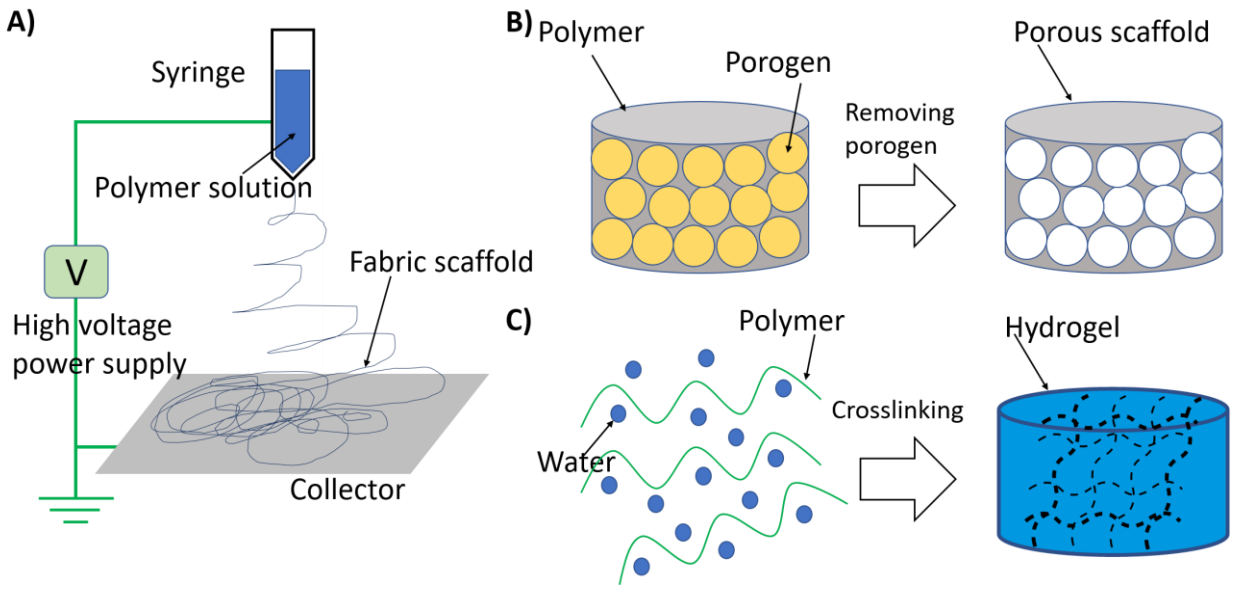

Figure 1.5 Typical methods for scaffolds fabrication. (A) Electrospinning for fabric scaffold. (B) Porogen leaching for porous scaffold. (C) Sol-gel crosslinking for hydrogel.

Hydrogels with microporous structures have drawn a lot of attention recently. Compared with bulk matrices structure, the microporous structure has the functions of promoting nutrient diffusion and providing space for cell spreading and proliferation. The fabrication methods for microporous structure include 3D printing, gas forming and porogen leaching. 3D printing can form hydrogel with complex structures. It has the ability to control the porosity, internal architecture and curved channels.[90] However, the hydrogel made by 3D printing cannot be injected which is important for cartilage tissue engineering. What's more, the high-cost of this method also limits the applications. The gas forming method is used to fabricate porous scaffolds and porous hydrogel.[91,92] However, the process conditions are not suitable for cell encapsulation. The frequently used methods for microporous hydrogel formation are using leachable porogen. After mixing with precursor solution and gelation, the porogen can be dissolved and leached after certain stimuli, such as EDTA for calcium crosslinked alginate microbeads and high temperature for physically crosslinked gelatin microparticles.[93,94] The leaching of gelatin physically crosslinked microparticles is biocompatible and friendly for *in vivo* applications. However, these gelatin microparticles cannot be used for cell laden, because the water/oil emulsion fabrication and sterilization processes make this method difficult for cell laden applications. Therefore, a new mild strategy for cell laden gelatin microgel fabrication is needed for

microporous hydrogel formation.

1.3 Crosslinking methods for hydrogels

1.3.1 Various crosslinking methods

a) Physically crosslinked hydrogels

Physically crosslinked hydrogels can be prepared at very mild conditions without using crosslinking agents that normally exhibit toxicity to cells or may affect the activity of bioactive molecules encapsulated in hydrogels.[95] There are many fabrication methods to prepare physically crosslinked hydrogels, such as ionic interactions and self-assembly (Figure 1.6).

I. Ionic crosslinking

The most representative hydrogels formed by ionic crosslinking is alginate hydrogel. The residues of mannuronic acid and glucuronic acid can be cross-linked by calcium ions.[96] This crosslinking method is frequently used to encapsulate cells and drugs due to the mild crosslinking environments (at room temperature and physiological pH).[97,98] Besides alginate, chitosan can also be crosslinked to form hydrogels by glycerol phosphate disodium salt.[99] Moreover, the sol-gel translation of this chitosan hydrogel can be adjusted to around 37 ^{o}C by increasing its deacetylation degree. Recently, one new ionic crosslinking method inspired by the mussel byssus adhesiveness has been used for biomedical applications.[100] The crosslinking site is formed by the bonding of catechol group and trivalent iron.[101] In addition to metallic ions crosslinking, hydrogel can be formed by complexation between polyanions, such as chitosan and polycations, such as dextran sulfate.[102] However, the ionically crosslinked hydrogels are usually unstable due to the exchange of ions *in vivo*.[103,104]

II. Self-assembly

Self-assembly protein-based hydrogels usually are physically crosslinked by the formation of triple helix structure and β-sheet structure. The mechanism involves hydrogen bonding and hydrophobic integration. During the crosslinking process, the polymers in aqueous solutions are assembled to form relatively stable crosslinking to become hydrogels. For example, collagen aqueous solution can form collagen hydrogel at neutral pH and 37 ^{o}C. The helix structure of collagen chain can assemble into nanofiber and be crosslinked together due to the increase of temperature at neutral pH.[105] The β-sheet rich structure of silk fibroin can work as the crosslinkage to inhibit the dissolution of polymer chains. Vortex or sonication can also be used to physically crosslink the silk fibroin to make hydrogels.[106]

Crystallization, the interaction between amphiphilic block and hydrogen bonding can also form physically crosslinked hydrogels. Poly(vinyl alcohol) (PVA) is a water soluble polymer that can be crosslinked by several freeze-thawing treatments. The PVA crystallites act as crosslinking sites in the 3D network.[107] However, this kind of PVA hydrogel can be used as the drug release hydrogel by loading BSA or other proteins,[108] rather than 3D cell culture. The amphiphilic block polymers can form hydrogels due to the self-assembly of hydrophobic parts of the polymers. For example, after hydrophobic modification, dextran, chitosan and other polysaccharides can form physically crosslinked hydrogels.[109,110] Hydrogen bonding can induce polyacrylic acid and polyethylene glycol to form a hydrogel, which usually is dependent on the pH.[111] Physical crosslinked gelatin hydrogel at a low temperature is also the result of hydrogen

bonding formation.[112]

Although the physically crosslinked methods normally have low toxicity, the fatal drawback of these non-covalently crosslinked hydrogels is unstable during *in vitro* cell culture or *in vivo* implantation. The mechanical support functions of scaffolds will lose if the crosslinking structures are not stable.[113]

b) Chemically crosslinked hydrogels

Chemically crosslinked hydrogels exhibit better mechanical property and stability compared with physically crosslinked hydrogels. There are many methods to prepare the chemically crosslinked hydrogels (Figure 1.6).

I. Crosslinking by complementary groups

Hydrogel-forming water-soluble polymers have many functional groups, such as OH, COOH and NH_2. The 3D network can be established by covalent bonding between these functional groups by Schiff base reaction or using glutaraldehyde and EDC/NHS.[114-116] But these chemical crosslinkers are cytotoxic to encapsulated cells.

II. Photopolymerization

Photopolymerization is a biocompatible crosslinking method to prepare hydrogels. This method has been widely used for hydrogel fabrications. The polymer materials are modified with photoreactive moieties, such as methacrylate or acrylate groups. The photoreactive polymer solution with photoinitiator can be crosslinked under UV light. The photoinitiators can generate free radicals that are transferred to the photoreactive carbon double bond groups in the modified polymers to start chain polymerization. This method is widely used because many biopolymers can be modified and a variety of cell types can be incorporated into the hydrogels.[117] Moreover, the gelation process can be controlled temporally and spatially, which means the hydrogels can be injected and polymerized in situ to fill the irregular cartilage defects. Therefore, this crosslinking method is ideal for cartilage tissue regeneration.

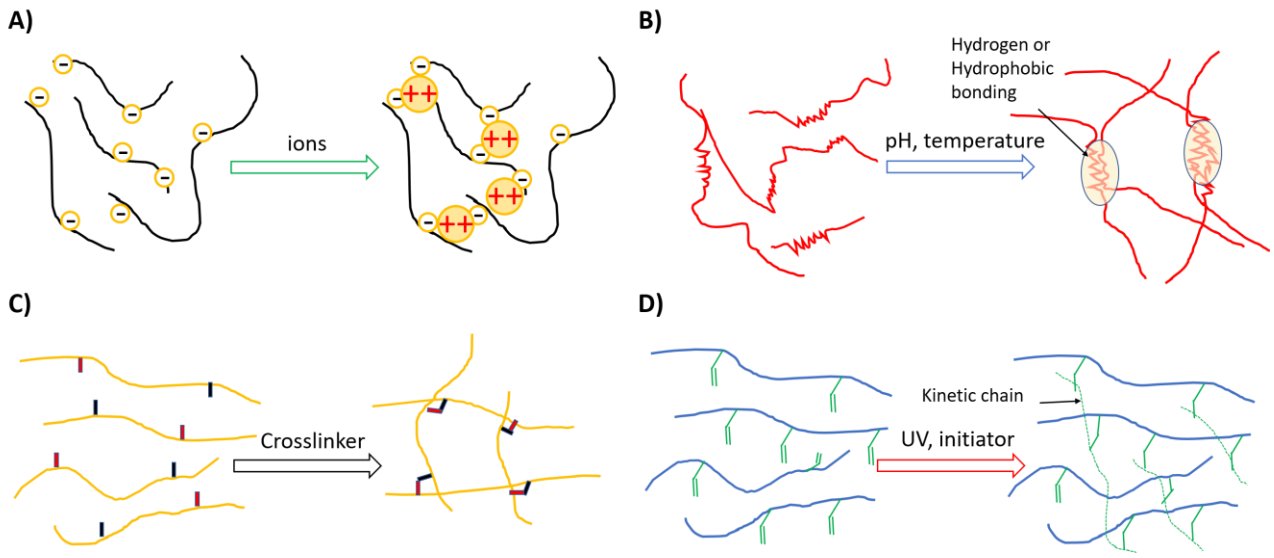

Figure 1.6 Illustration of hydrogel crosslinking mechanism. (A) Ionic crosslinking, (B) Self-assembly, (C) Chemical Crosslinking by complementary groups and (D) Photopolymerization.

1.3.2 Mechanism of photopolymerization

Photopolymerization exhibits many advantages, such as injectability, quick gelation and easy incorporation with cells and other chemistries. However, there is a limitation of this method. During crosslinking the free radicals will be generated. Free radicals can attack cell membrane to induce cell death. However, this effect is dosage dependent. Studies have reported that mild condition of photocrosslinking is biocompatible, which can be realized easily by decreasing the light energy and amount of photoinitiator.[118,119] It has also been reported that high density of methacrylate groups can protect encapsulated cells.[120] This section summarizes all the factors involved in the process of photopolymerization.

a) Photocrosslinkable polymers

Photocrosslinking method includes introduction of photoreactive vinyl groups in the polymer, and exposure to UV light with the presence of photoinitiators. Synthetic material like PEG and many kinds of natural polymers have been studied to fabricate the hydrogels by photopolymerization. Poly (ethylene glycol) diacrylate (PEGDA) has been synthesized by the modification with acryloyl chloride under the nitrogen environments.[121] However, PEGDA hydrogel is cell nonadhesive and not able to absorb proteins. And due to the non-degradability, PEGDA hydrogel usually works as a nondegradable control in short-term *in vitro* cell culture. HA is the commonly used polymer for cartilage tissue engineering. Photoreactive HA (HAMA) can be obtained by the modification with glycidyl methacrylate.[122] The previous studies have shown that HAMA hydrogel can promote the maintenance of the chondrogenic phenotype of chondrocytes, chondrogenic differentiation of MSC and secretion of cartilaginous matrices both *in vitro* and *in vivo*.[123,124]. Similarly, chondroitin sulfate and chitosan can also be grafted with vinyl groups by reaction with glycidyl methacrylate for photopolymerization.[125] The other modification method is to react the amino groups in polymers with methacrylic acid. Gelatin methacrylate macromer is usually prepared by this method, which can be very easily conducted in a neutral aqueous solution.[126] In this dissertation, gelatin methacrylate (GelMA) was used due to the easy modification method and the good biocompatibility of this macromer.[127] Furthermore, it is suitable to study the cell-matrix interaction due to the bioactive motifs, such as RGD in the polymer chains.

b) Photoinitiators

There are many kinds of photoinitiators for polymerizing hydrogels, such as 2-hydroxy-1-[4-(hydroxy ethoxy)phenyl]-2-methyl-1-propanone(I2959), 1-hydroxycyclohexyl phenyl ketone (HPK) and 2,2-dimethoxy-2-phenylacetophenone (I651).[128] The initiator as a small molecular, before or after UV exposure, can be uptaken by cells. William et al. have compared the toxicity of I2959, HPK and I651 photoinitiators. The results have shown that even without UV exposure, the molecules with high concentration can affect the cell viability by WST-1 assay. I2959 has less toxicity compared with HPK and I651. Cultivation of cells with 0.1% I2959 shows almost no effects on bovine chondrocytes viability, but 80% cell survival for human fetal osteoblasts. I2959 can maintain high cell survival even after 7 minutes exposure to UV light. The reason of the toxicity of initiators is thought partly due to the hydrophobicity of the molecule.[129] Hydrophobicity can increase the permeability through phospholipid bilayers of cellular membranes. The concentration of photoinitiators should be controlled in a way that the generated heating and free radicals will not show toxicity to cells and surrounding tissues.[130] In this study, I2959 was used as the photoinitiator due to the low cytotoxicity.

c) UV energy

UV irradiation conditions should be optimized because UV light may affect cell viability.[131] UV intensity can be controlled by setting the UV energy density of the UV crosslinker machine or by adjusting the UV exposure time when the fixed power machine is used.[132] The UV irradiation conditions can also affect the polymerization degree.[133]

d) Free radicals

Photoinitiator can generate free radicals once exposure to UV light. The free radicals can trigger the radical polymerization of methacrylate modified polymers. At the same time, free radical can attack the cells to break cell membrane and induce generation of intracellular reactive oxygen species. The reactive oxygen species can damage proteins and DNA.[134] Therefore, cells may die if the reactive oxygen species increases to a certain level.[135] Although this potential drawback exists in photopolymerization system, the toxicity can be adjusted by changing the concentration of photoinitiator or the exposure UV energy. For example, Bryant et al. have shown that using I2959 activation by 365 nm UV light can be well tolerated by fibroblast and chondrocyte.[118] Poly(ethylene glycol) hydrogel polymerized by this system has been used for the 3D culture of chondrocytes, osteoblasts, mesenchymal and embryonic stem cells.[86,136-138] Therefore, the negative effects of free radicals are tolerable if the amount of photoinitiator and UV exposure energy are well controlled.

1.4 The effect of hydrogel properties on chondrocyte functions

The challenges for cartilage tissue engineering include the proliferation of cells, maintenance of chondrogenic phenotype and mechanical property of the engineered constructs.[139] Hydrogel is 3D crosslinked polymer network with abundant water absorption, which is an ideal cell carrier due to the biomimetic microenvironment. The biomechanical and biochemical properties of hydrogel, the crosslinking density of hydrogel matrices and the microporous structure of hydrogels can affect cell functions. Chondrocytes may lose chondrogenic phenotype during 2D cell expansion culture. Therefore, many studies have employed growth factors or cell condensation culture to restore the chondrogenic function of dedifferentiated chondrocytes.

1.4.1 The effect of biomechanical property of hydrogel

It has been reported that the stiffness of 2D surface affects the differentiation direction of MSC.[20] Low stiffness promotes the adipogenic differentiation, while high stiffness is beneficial for osteogenesis. Not only the stiffness of matrices, stress relaxation of hydrogel matrices also has some effects on osteogenic and adipogenic differentiation.[140] Quick relaxation promotes the osteogenesis while slow relaxation is beneficial for adipogenesis.[141] Because of the importance of the biomechanical property of hydrogels, many literatures have reported the influence of matrix stiffness on the chondrogenic phenotype. For example, PEG hydrogels with different compressive moduli have been fabricated by the various concentration of macromers and used for chondrocytes culture.[142] HA hydrogels and alginate hydrogels with gradient Young’s modulus have also been prepared by changing the macromer concentration.[143,144] Chondrocytes can response to the stiffness of the matrices by mechano-transductive pathways and show different behaviors.

Besides stiffness, degradation property and crosslinking density can also affect chondrocyte functions.

1.4.2 The effect of biochemical property of hydrogel

Besides the biomechanical property, chemical composition of hydrogel precursors can highly affect the cell behaviors including viability, adhesion, proliferation and differentiation. For example, the hydrophobic and hydrophilic property, charge of environment and cell active motifs are dependent on the biochemical property of hydrogels. Synthetic polymer formed hydrogels usually have a hydrophobic property which inhibits the cell attachment and other activities. The native ECM of normal tissue is comprised of collagen, laminin, fibronectin and aggrecan. The ECM provides favorable biochemical stimuli, such as RGD for certain cell functions. The adhesion sites like RGD tripeptide and the receptors on cell membrane constitute the whole recognition system for cell adhesion, migration, proliferation and differentiation.[145] Therefore, RGD peptides sequence is very important for cell activity. It has been demonstrated that RGD can promote the survival of MSC in PEG hydrogels and induce the chondrogenic differentiation.[22] RGD ligands have also been reported to increase the chondrogenic gene expression when the matrices are loaded with dynamic mechanical force.[146] The results indicate the RGD ligands are necessary for cell-matrix interactions.[136] Moreover, different RGD density can affect the redifferentiation of chondrocytes.[147] For chondrocyte culture, HA can bond to the CD 44 receptor on cell membrane to affect both chondrocyte survival pathway and apoptotic pathway.[148] Therefore, the biochemical property of hydrogel is critical for cell activities and functions.

1.4.3 The effect of pore structure of hydrogel

The structure of bulk hydrogels is dense polymers with absorbed water and nano-size pores within the network.[149] These porous structures allow the exchange of nutrition and waste. However, these pores are too small to promote cell proliferation and ECM diffusion. This is the main reason that microporous scaffolds are required to offer better nutrition supply and to promote cell proliferation.[150] Collagen sponges with open and interconnective microporous structure have been prepared by mixing with ice particles.[151] The effects of different pore structure and pore size have been studied for cell culture and tissue engineering.[58,63] Hydrogels and porous scaffolds have been compared for culture of chondrocytes.[152] Chondrocytes exhibit different morphology, chondrogenesis gene expression and production of ECM in hydrogels and porous scaffolds. Proliferation and collagen type I gene expression are enhanced but the aggrecan gene expression is down-regulated in sponge scaffolds.[152] However, the porous scaffold has shown better functions on chondrogenic differentiation of MSC due to cell aggregation after proliferation and improved cell-cell interaction.[153] Many researchers have combined the microporous structure of pre-made 3D scaffolds with injectable hydrogels to prepare microporous hydrogels.[154,155] They believe the microporous structure should be good for nutrition diffusion and can provide space for cell proliferation. For example, physically crosslinked gelatin microbeads have been fabricated and encapsulated in alginate hydrogels for cartilage tissue engineering.[156] The results indicate the microporous structures promote cell proliferation and up-regulate gene expression of chondrocytes.[157] However, during the gradually dissolution and diffusion of porogen materials, different porogen materials and bulk gel materials will result in different microenvironments in the micropores and bulk hydrogels. To elucidate the effect of microstructures, the same porogen materials and bulk hydrogel matrices are desirable to eliminate the effect

from the material difference.

1.5 Motivation, objectives and outline

1.5.1 Motivation and objectives

The biomechanical and biochemical properties of hydrogels affect the cell behaviors, such as attachment, migration, proliferation and differentiation. Stiffness as an important biomechanical factor has some effects on stem cell differentiation and the maintenance of chondrocyte phenotype. In order to promote the chondrogenesis of chondrocytes encapsulated in hydrogels, the properties of hydrogels should be studied and optimized to meet the requirement of an ideal scaffold and to offer the best environment for the chondrocytes. There are several studies to explore the influence of stiffness on chondrocytes functions. The materials used for hydrogels include PEG, agarose and HA. These designs cannot mimic the microenvironment of native cartilage. Firstly, the concentration is different which may affect the nutrition diffusions and mesh density around the chondrocytes. Secondly, the materials used in previous studies do not possess the RGD motifs that are important to control cell functions and to translate mechanical stimulus to cells. For example, synthetic polymers, such as PEG shows better influence on chondrocytes after modification with RGD motifs.[136] On the other hand, gelatin based hydrogels contain RGD motifs and can maintain high cell viability and activity due to their bioactivity. Therefore, biopolymers with RGD sequence should be used to prepare the hydrogels. It is meaningful to study the influence of hydrogel stiffness while excluding the other factors like mass concentrations and RGD density. To reach such purpose, the hydrogels with different stiffness while same RGD density should be used. In this study, the same gelatin concentration will be used to prepare the gelatin methacrylate hydrogels. Different stiffness will be achieved by changing the degree of methacrylate while keeping gelatin concentration at the same level.

Gelatin based hydrogels are suitable to mimic the microenvironment of native ECM. But the degradation speed and mechanical properties limit the clinical applications, especially for long-term implantation. Several methods have been used to improve the mechanical properties, such as increasing the crosslinker density or the concentration of precursor solutions. These approaches may affect cell viability due to the crosslinker toxicity and the viscosity of solutions. For injectable hydrogels, the high viscosity solution will hurt cells by high shear forces during mixing and injections, at the same time, increase the difficulty for operations.[158] An effective way is desirable to increase the mechanical property, to prolong the degradation time and to decrease the viscosity of the precursor solution. In this study, the double methacrylate modified gelatin will be used to solve these limitations. This macromer has a high amount of photoreactive vinyl groups allowing the high crosslinking degree which should improve the mechanical property and degradation profiles. Furthermore, the second modification can decrease the viscosity of the solution by decreasing the hydrogen bonding. By using the GelMA and GelMAGMA hydrogels, the influence of crosslinking density on chondrocytes proliferation and phenotype will be investigated.

Although gelatin hydrogels have shown excellent effects on chondrocyte functions, such as promoting the chondrogenic gene expression and chondrogenic ECM secretion, cell proliferation in hydrogel is hampered. Moreover, the assembly of secreted ECM is inhibited by the crosslinked polymer network. Porous structure of scaffold has been reported to promote cell proliferation by providing the space and enhancing nutrition diffusion. Introduction of microporous structures in hydrogels to make microporous gelatin hydrogels is an effective strategy to solve the problems. In this study, a useful method to prepare

microporous gelatin hydrogels will be proposed. The microporous gelatin hydrogels will be used for 3D culture of chondrocytes and comparison with gelatin hydrogels without microporous structures.

1.5.2 Outline

In this study, gelatin hydrogels with different degrees of methacrylation, mechanical properties and microporous structures will be prepared for chondrocyte culture and cartilage regeneration to explore the influence of biomechanical properties and microporous structures on chondrocytes functions.

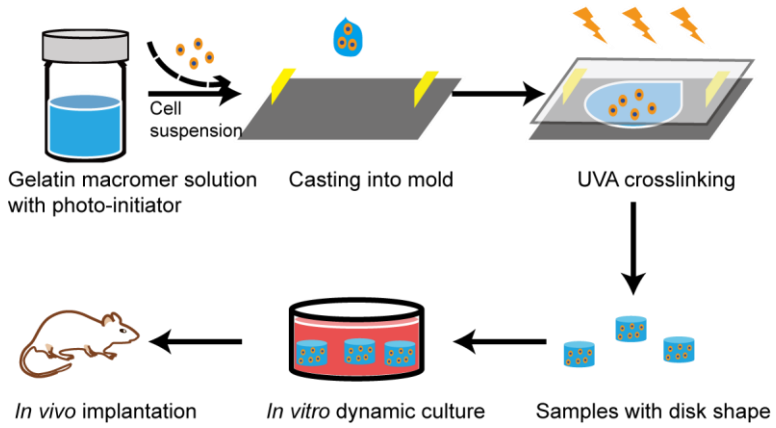

Figure 1.7 Preparation scheme of cell-laden gelatin hydrogels by photopolymerization and 3D culture of chondrocytes *in vitro* and *in vivo*.

In chapter 2, GelMA hydrogels with various stiffness were fabricated by using macromer with different modification degrees. The same concentration of gelatin was used to exclude the effects of biochemical, such as RGD density. To test the stiffness of local matrices accurately, atomic force microscope (AFM) was used to measure the stiffness. After two weeks culture, the morphology, gene expression and ECM secretion of chondrocytes encapsulated in different stiffness hydrogels were compared.

In chapter 3, glycidyl methacrylate was used to double modify the GelMA macromer with high functionalization degree. The mechanical properties including storage modulus and degradation were studied. The influence of crosslinking density on chondrocyte proliferation and phenotype was compared by using GelMA and GelMAGMA hydrogels.

In chapter 4, physical crosslinked gelatin microgels with cubic shapes were prepared by mesh-cutting method. These microcubes were mixed in GelMAGMA macromer solution before UV crosslinking. The microcubes were dissolved to induce microcavities after *in vitro* culture at 37 ºC or *in vivo* implantation. Cell-laden GelMAGMA bulk hydrogel, cell-laden GelMAGMA bulk hydrogel with pure gelatin microcubes and GelMAGMA bulk hydrogel with cell-laden gelatin microcubes were designed and studied *in vitro* and *in vivo* environments to investigate the effect of microporous structures on cell functions.

In chapter 5, the conclusions and prospects of this dissertation are summarized.

1.6 References

1. Temenoff, J.S.; Mikos, A.G. Review: Tissue engineering for regeneration of articular cartilage. *Biomaterials* **2000**, *21*, 431-440.
2. Ghosh, P. Nonsteroidal anti-inflammatory drugs and chondroprotection. A review of the evidence. *Drugs* **1993**, *46*, 834.
3. Mitchell, N.; Shepard, N. The resurfacing of adult rabbit articular cartilage by multiple perforations through the subchondral bone. *JBJS* **1976**, *58*, 230-233.
4. Johnson, L.L. Arthroscopic abrasion arthroplasty historical and pathologic perspective: Present status. *Arthroscopy: The Journal of Arthroscopic & Related Surgery* **1986**, *2*, 54-69.
5. Rodrigo, J.; Steadman, J.; Silliman, J.; Fulstone, H. Improvement of full-thickness chondral defect healing in the human knee after debridement and microfracture using continuous passive motion. *Am J Knee Surg* **1994**, *7*, 109-116.
6. Gobbi, A.; Francisco, R.A.; Lubowitz, J.H.; Allegra, F.; Canata, G. Osteochondral lesions of the talus: Randomized controlled trial comparing chondroplasty, microfracture, and osteochondral autograft transplantation. *Arthroscopy: The Journal of Arthroscopic & Related Surgery* **2006**, *22*, 1085-1092.
7. Mithoefer, K.; McAdams, T.; Williams, R.J.; Kreuz, P.C.; Mandelbaum, B.R. Clinical efficacy of the microfracture technique for articular cartilage repair in the knee: An evidence-based systematic analysis. *The American journal of sports medicine* **2009**, *37*, 2053-2063.
8. Chen, S.; Zhang, Q.; Kawazoe, N.; Chen, G. Effect of high molecular weight hyaluronic acid on chondrocytes cultured in collagen/hyaluronic acid porous scaffolds. *RSC Adv.* **2015**, *5*, 94405-94410.
9. Chen, G.; Sato, T.; Ushida, T.; Hirochika, R.; Tateishi, T. Redifferentiation of dedifferentiated bovine chondrocytes when cultured in vitro in a PLGA–collagen hybrid mesh. *FEBS Lett.* **2003**, *542*, 95-99.
10. Pittenger, M.F.; Mackay, A.M.; Beck, S.C.; Jaiswal, R.K.; Douglas, R.; Mosca, J.D.; Moorman, M.A.; Simonetti, D.W.; Craig, S.; Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *science* **1999**, *284*, 143-147.
11. Zuk, P.A.; Zhu, M.; Mizuno, H.; Huang, J.; Futrell, J.W.; Katz, A.J.; Benhaim, P.; Lorenz, H.P.; Hedrick, M.H. Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Eng.* **2001**, *7*, 211-228.
12. De Bari, C.; Dell'Accio, F.; Tylzanowski, P.; Luyten, F.P. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis & Rheumatism* **2001**, *44*, 1928-1942.
13. Sridhar, B.V.; Doyle, N.R.; Randolph, M.A.; Anseth, K.S. Covalently tethered TGF‐β1 with encapsulated chondrocytes in a PEG hydrogel system enhances extracellular matrix production. *J Biomed Mater Res A* **2014**, *102*, 4464-4472.
14. Matsiko, A.; Levingstone, T.J.; Gleeson, J.P.; O'brien, F.J. Incorporation of TGF‐β3 within collagen‐hyaluronic acid scaffolds improves their chondrogenic potential. *Advanced healthcare materials* **2015**, *4*, 1175-1179.
15. Sittinger, M.; Reitzel, D.; Dauner, M.; Hierlemann, H.; Hammer, C.; Kastenbauer, E.; Planck, H.; Burmester, G.; Bujia, J. Resorbable polyesters in cartilage engineering: Affinity

and biocompatibility of polymer fiber structures to chondrocytes. *J Biomed Mater Res A* **1996**, *33*, 57-63.

16. Hutmacher, D.W. Scaffolds in tissue engineering bone and cartilage. *Biomaterials* **2000**, *21*, 2529-2543.
17. Godbey, W.; Atala, A. In vitro systems for tissue engineering. *Ann. N.Y. Acad. Sci.* **2002**, *961*, 10-26.
18. Bryant, S.J.; Anseth, K.S. Controlling the spatial distribution of ECM components in degradable PEG hydrogels for tissue engineering cartilage. *J Biomed Mater Res A* **2003**, *64*, 70-79.
19. Li, W.J.; Laurencin, C.T.; Caterson, E.J.; Tuan, R.S.; Ko, F.K. Electrospun nanofibrous structure: A novel scaffold for tissue engineering. *Journal of biomedical materials research* **2002**, *60*, 613-621.
20. Engler, A.J.; Sen, S.; Sweeney, H.L.; Discher, D.E. Matrix elasticity directs stem cell lineage specification. *Cell* **2006**, *126*, 677-689.
21. Benoit, D.S.; Schwartz, M.P.; Durney, A.R.; Anseth, K.S. Small functional groups for controlled differentiation of hydrogel-encapsulated human mesenchymal stem cells. *Nat. Mater.* **2008**, *7*, 816-823.
22. Salinas, C.N.; Anseth, K.S. The enhancement of chondrogenic differentiation of human mesenchymal stem cells by enzymatically regulated RGD functionalities. *Biomaterials* **2008**, *29*, 2370-2377.
23. Holy, C.E.; Cheng, C.; Davies, J.E.; Shoichet, M.S. Optimizing the sterilization of plga scaffolds for use in tissue engineering. *Biomaterials* **2000**, *22*, 25-31.
24. Drury, J.L.; Mooney, D.J. Hydrogels for tissue engineering: Scaffold design variables and applications. *Biomaterials* **2003**, *24*, 4337-4351.
25. Browning, M.; Cereceres, S.; Luong, P.; Cosgriff‐Hernandez, E. Determination of the in vivo degradation mechanism of pegda hydrogels. *J Biomed Mater Res A* **2014**, *102*, 4244-4251.
26. Peng, H.; Varanasi, S.; Wang, D.K.; Blakey, I.; Rasoul, F.; Symons, A.; Hill, D.J.; Whittaker, A.K. Synthesis, swelling, degradation and cytocompatibility of crosslinked PLLA-PEG-PLLA networks with short PLLA blocks. *Eur. Polym. J.* **2016**, *84*, 448-464.
27. Musumeci, G.; Loreto, C.; Castorina, S.; Imbesi, R.; Leonardi, R.; Castrogiovanni, P. New perspectives in the treatment of cartilage damage. Poly (ethylene glycol) diacrylate (pegda) scaffold. A review. *Italian Journal of Anatomy and Embryology* **2013**, *118*, 204.
28. Nie, L.; Zhang, G.; Hou, R.; Xu, H.; Li, Y.; Fu, J. Controllable promotion of chondrocyte adhesion and growth on PVA hydrogels by controlled release of TGF-β1 from porous plga microspheres. *Colloids Surf., B* **2015**, *125*, 51-57.
29. Makadia, H.K.; Siegel, S.J. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers* **2011**, *3*, 1377-1397.
30. Baldwin, A.D.; Kiick, K.L. Polysaccharide‐modified synthetic polymeric biomaterials. *Pept. Sci.* **2010**, *94*, 128-140.
31. Lee, C.H.; Singla, A.; Lee, Y. Biomedical applications of collagen. *Int. J. Pharm.* **2001**, *221*, 1-22.
32. Smidsrød, O.; Skja, G. Alginate as immobilization matrix for cells. *Trends Biotechnol.* **1990**, *8*, 71-78.

33. Suh, J.-K.F.; Matthew, H.W. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: A review. *Biomaterials* **2000**, *21*, 2589-2598.
34. Pariente, J.L.; Kim, B.S.; Atala, A. In vitro biocompatibility assessment of naturally derived and synthetic biomaterials using normal human urothelial cells. *Journal of biomedical materials research* **2001**, *55*, 33-39.
35. Rinaudo, M. Main properties and current applications of some polysaccharides as biomaterials. *Polym. Int.* **2008**, *57*, 397-430.
36. Manna, F.; Dentini, M.; Desideri, P.; De Pita, O.; Mortilla, E.; Maras, B. Comparative chemical evaluation of two commercially available derivatives of hyaluronic acid (hylaform® from rooster combs and restylane® from streptococcus) used for soft tissue augmentation. *Journal of the European Academy of Dermatology and Venereology* **1999**, *13*, 183-192.
37. Jakobsen, R.B.; Shahdadfar, A.; Reinholt, F.P.; Brinchmann, J.E. Chondrogenesis in a hyaluronic acid scaffold: Comparison between chondrocytes and MSC from bone marrow and adipose tissue. *Knee Surgery, Sports Traumatology, Arthroscopy* **2010**, *18*, 1407-1416.
38. Savani, R.C.; Wang, C.; Yang, B.; Zhang, S.; Kinsella, M.G.; Wight, T.N.; Stern, R.; Nance, D.M.; Turley, E.A. Migration of bovine aortic smooth muscle cells after wounding injury. The role of hyaluronan and rhamm. *Journal of Clinical Investigation* **1995**, *95*, 1158.
39. Leppilahti, M.; Hellström, P.; Tammela, T.L. Effect of diagnostic hydrodistension and four intravesical hyaluronic acid instillations on bladder ICAM-1 intensity and association of ICAM-1 intensity with clinical response in patients with interstitial cystitis. *Urology* **2002**, *60*, 46-51.
40. Kim, T.G.; Chung, H.J.; Park, T.G. Macroporous and nanofibrous hyaluronic acid/collagen hybrid scaffold fabricated by concurrent electrospinning and deposition/leaching of salt particles. *Acta Biomater.* **2008**, *4*, 1611-1619.
41. Baier Leach, J.; Bivens, K.A.; Patrick Jr, C.W.; Schmidt, C.E. Photocrosslinked hyaluronic acid hydrogels: Natural, biodegradable tissue engineering scaffolds. *Biotechnol. Bioeng.* **2003**, *82*, 578-589.
42. Baeurle, S.; Kiselev, M.; Makarova, E.; Nogovitsin, E. Effect of the counterion behavior on the frictional–compressive properties of chondroitin sulfate solutions. *Polymer* **2009**, *50*, 1805-1813.
43. Zhu, Y.; Wu, H.; Sun, S.; Zhou, T.; Wu, J.; Wan, Y. Designed composites for mimicking compressive mechanical properties of articular cartilage matrix. *J. Mech. Behav. Biomed. Mater.* **2014**, *36*, 32-46.
44. Levett, P.A.; Melchels, F.P.; Schrobback, K.; Hutmacher, D.W.; Malda, J.; Klein, T.J. A biomimetic extracellular matrix for cartilage tissue engineering centered on photocurable gelatin, hyaluronic acid and chondroitin sulfate. *Acta Biomater.* **2014**, *10*, 214-223.
45. JOHNSON, F.A.; CRAIG, D.Q.; MERCER, A.D. Characterization of the block structure and molecular weight of sodium alginates. *J. Pharm. Pharmacol.* **1997**, *49*, 639-643.
46. Haug, A. Fractionation of alginic acid. MUNKSGAARD INT PUBL LTD 35 NORRE SOGADE, PO BOX 2148, DK-1016 COPENHAGEN, DENMARK: 1959; Vol. 13, pp 601-603.
47. Chang, S.C.; Tobias, G.; Roy, A.K.; Vacanti, C.A.; Bonassar, L.J. Tissue engineering of autologous cartilage for craniofacial reconstruction by injection molding. *Plastic and*

reconstructive surgery **2003**, *112*, 793-799.

48. George, M.; Abraham, T.E. Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan—a review. *J. Controlled Release* **2006**, *114*, 1-14.
49. Otterlei, M.; Østgaard, K.; Skjåk-Bræk, G.; Smidsrød, O.; Soon-Shiong, P.; Espevik, T. Induction of cytokine production from human monocytes stimulated with alginate. *Journal of Immunotherapy* **1991**, *10*, 286-291.
50. LeRoux, M.A.; Guilak, F.; Setton, L.A. Compressive and shear properties of alginate gel: Effects of sodium ions and alginate concentration. *Journal of biomedical materials research* **1999**, *47*, 46-53.
51. Al-Shamkhani, A.; Duncan, R. Radioiodination of alginate via covalently-bound tyrosinamide allows monitoring of its fate in vivo. *Journal of bioactive and compatible polymers* **1995**, *10*, 4-13.
52. Baruch, L.; Machluf, M. Alginate–chitosan complex coacervation for cell encapsulation: Effect on mechanical properties and on long‐term viability. *Biopolymers* **2006**, *82*, 570-579.
53. Zimmermann, A.C.; Mecabô, A.; Fagundes, T.; Rodrigues, C.A. Adsorption of cr (vi) using fe-crosslinked chitosan complex (ch-fe). *J. Hazard. Mater.* **2010**, *179*, 192-196.
54. Tan, H.; Wan, L.; Wu, J.; Gao, C. Microscale control over collagen gradient on poly (l-lactide) membrane surface for manipulating chondrocyte distribution. *Colloids Surf., B* **2008**, *67*, 210-215.
55. Heim, M.; Römer, L.; Scheibel, T. Hierarchical structures made of proteins. The complex architecture of spider webs and their constituent silk proteins. *Chem. Soc. Rev.* **2010**, *39*, 156-164.
56. Mueller, S.M.; Shortkroff, S.; Schneider, T.O.; Breinan, H.A.; Yannas, I.V.; Spector, M. Meniscus cells seeded in type I and type II collagen–GAG matrices in vitro. *Biomaterials* **1999**, *20*, 701-709.
57. Chen, S.; Zhang, Q.; Nakamoto, T.; Kawazoe, N.; Chen, G. Highly active porous scaffolds of collagen and hyaluronic acid prepared by suppression of polyion complex formation. *J Mater Chem B* **2014**, *2*, 5612-5619.
58. Zhang, Q.; Lu, H.; Kawazoe, N.; Chen, G. Pore size effect of collagen scaffolds on cartilage regeneration. *Acta Biomater.* **2014**, *10*, 2005-2013.
59. Eyre, D.R. Collagen: Molecular diversity in the body's protein scaffold. *Science* **1980**, *207*, 1315-1322.
60. Zhang, Z.; Li, G.; Shi, B. Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. *JOURNAL-SOCIETY OF LEATHER TECHNOLOGISTS AND CHEMISTS* **2006**, *90*, 23.
61. Hersel, U.; Dahmen, C.; Kessler, H. RGD modified polymers: Biomaterials for stimulated cell adhesion and beyond. *Biomaterials* **2003**, *24*, 4385-4415.
62. Gómez-Guillén, M.; Giménez, B.; López-Caballero, M.a.; Montero, M. Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids* **2011**, *25*, 1813-1827.
63. Chen, S.; Zhang, Q.; Nakamoto, T.; Kawazoe, N.; Chen, G. Gelatin scaffolds with controlled pore structure and mechanical property for cartilage tissue engineering. *Tissue Eng Part C Methods* **2016**, *22*, 189-198.

64. Van Vlierberghe, S.; Dubruel, P.; Schacht, E. Biopolymer-based hydrogels as scaffolds for tissue engineering applications: A review. *Biomacromolecules* **2011**, *12*, 1387-1408.
65. Mosesson, M. Fibrinogen and fibrin structure and functions. *Journal of Thrombosis and Haemostasis* **2005**, *3*, 1894-1904.
66. Ramaswamy, S.; Wang, D.A.; Fishbein, K.W.; Elisseeff, J.H.; Spencer, R.G. An analysis of the integration between articular cartilage and nondegradable hydrogel using magnetic resonance imaging. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **2006**, *77*, 144-148.
67. Schense, J.C.; Hubbell, J.A. Cross-linking exogenous bifunctional peptides into fibrin gels with factor XIIIa. *Bioconjugate Chem.* **1999**, *10*, 75-81.
68. Dare, E.V.; Griffith, M.; Poitras, P.; Kaupp, J.A.; Waldman, S.D.; Carlsson, D.J.; Dervin, G.; Mayoux, C.; Hincke, M.T. Genipin cross-linked fibrin hydrogels for in vitro human articular cartilage tissue-engineered regeneration. *Cells Tissues Organs* **2009**, *190*, 313-325.
69. Hsieh, F.-Y.; Tao, L.; Wei, Y.; Hsu, S.-h. A novel biodegradable self-healing hydrogel to induce blood capillary formation. *NPG Asia Materials* **2017**, *9*, e363.
70. Altman, G.H.; Diaz, F.; Jakuba, C.; Calabro, T.; Horan, R.L.; Chen, J.; Lu, H.; Richmond, J.; Kaplan, D.L. Silk-based biomaterials. *Biomaterials* **2003**, *24*, 401-416.
71. Uebersax, L.; Merkle, H.P.; Meinel, L. Insulin-like growth factor I releasing silk fibroin scaffolds induce chondrogenic differentiation of human mesenchymal stem cells. *J. Controlled Release* **2008**, *127*, 12-21.
72. Gobin, A.S.; Rhea, R.; Newman, R.A.; Mathur, A.B. Silk-fibroin-coated liposomes for long-term and targeted drug delivery. *Int. J. Nanomed.* **2006**, *1*, 81.
73. Correia, C.; Bhumiratana, S.; Yan, L.-P.; Oliveira, A.L.; Gimble, J.M.; Rockwood, D.; Kaplan, D.L.; Sousa, R.A.; Reis, R.L.; Vunjak-Novakovic, G. Development of silk-based scaffolds for tissue engineering of bone from human adipose-derived stem cells. *Acta Biomater.* **2012**, *8*, 2483-2492.
74. Yucel, T.; Cebe, P.; Kaplan, D.L. Vortex-induced injectable silk fibroin hydrogels. *Biophys. J.* **2009**, *97*, 2044-2050.
75. Wang, X.; Kluge, J.A.; Leisk, G.G.; Kaplan, D.L. Sonication-induced gelation of silk fibroin for cell encapsulation. *Biomaterials* **2008**, *29*, 1054-1064.
76. Lv, Q.; Hu, K.; Feng, Q.; Cui, F. Fibroin/collagen hybrid hydrogels with crosslinking method: Preparation, properties, and cytocompatibility. *J Biomed Mater Res A* **2008**, *84*, 198-207.
77. Montjovent, M.-O.; Mathieu, L.; Hinz, B.; Applegate, L.L.; Bourban, P.-E.; Zambelli, P.-Y.; Månson, J.-A.; Pioletti, D.P. Biocompatibility of bioresorbable poly (L-lactic acid) composite scaffolds obtained by supercritical gas foaming with human fetal bone cells. *Tissue Eng.* **2005**, *11*, 1640-1649.
78. Liu, X.; Ma, P.X. Phase separation, pore structure, and properties of nanofibrous gelatin scaffolds. *Biomaterials* **2009**, *30*, 4094-4103.
79. Liao, C.J.; Chen, C.F.; Chen, J.H.; Chiang, S.F.; Lin, Y.J.; Chang, K.Y. Fabrication of porous biodegradable polymer scaffolds using a solvent merging/particulate leaching method. *Journal of biomedical materials research* **2002**, *59*, 676-681.
80. Chen, V.J.; Ma, P.X. Nano-fibrous poly (L-lactic acid) scaffolds with interconnected spherical macropores. *Biomaterials* **2004**, *25*, 2065-2073.

81. Lu, H.; Ko, Y.-G.; Kawazoe, N.; Chen, G. Cartilage tissue engineering using funnel-like collagen sponges prepared with embossing ice particulate templates. *Biomaterials* **2010**, *31*, 5825-5835.
82. Ero-Phillips, O.; Jenkins, M.; Stamboulis, A. Tailoring crystallinity of electrospun PLLA fibres by control of electrospinning parameters. *Polymers* **2012**, *4*, 1331-1348.
83. Ma, Z.; Kotaki, M.; Inai, R.; Ramakrishna, S. Potential of nanofiber matrix as tissue-engineering scaffolds. *Tissue Eng.* **2005**, *11*, 101-109.
84. Vasita, R.; Katti, D.S. Nanofibers and their applications in tissue engineering. *Int. J. Nanomed.* **2006**, *1*, 15.
85. Beachley, V.; Wen, X. Polymer nanofibrous structures: Fabrication, biofunctionalization, and cell interactions. *Prog. Polym. Sci.* **2010**, *35*, 868-892.
86. Chung, C.; Burdick, J.A. Influence of three-dimensional hyaluronic acid microenvironments on mesenchymal stem cell chondrogenesis. *Tissue Eng. Part A* **2008**, *15*, 243-254.
87. Aisenbrey, E.; Bryant, S. Mechanical loading inhibits hypertrophy in chondrogenically differentiating hMSCs within a biomimetic hydrogel. *J Mater Chem B* **2016**, *4*, 3562-3574.
88. Awad, H.A.; Wickham, M.Q.; Leddy, H.A.; Gimble, J.M.; Guilak, F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials* **2004**, *25*, 3211-3222.
89. Callahan, L.A.S.; Ganios, A.M.; Childers, E.P.; Weiner, S.D.; Becker, M.L. Primary human chondrocyte extracellular matrix formation and phenotype maintenance using RGD-derivatized PEGDM hydrogels possessing a continuous young's modulus gradient. *Acta Biomater.* **2013**, *9*, 6095-6104.
90. Chia, H.N.; Wu, B.M. Recent advances in 3D printing of biomaterials. *Journal of biological engineering* **2015**, *9*, 4.
91. Mooney, D.J.; Baldwin, D.F.; Suh, N.P.; Vacanti, J.P.; Langer, R. Novel approach to fabricate porous sponges of poly (D, L-lactic-co-glycolic acid) without the use of organic solvents. *Biomaterials* **1996**, *17*, 1417-1422.
92. Neffe, A.T.; Pierce, B.F.; Tronci, G.; Ma, N.; Pittermann, E.; Gebauer, T.; Frank, O.; Schossig, M.; Xu, X.; Willie, B.M. One step creation of multifunctional 3D architectured hydrogels inducing bone regeneration. *Adv. Mater.* **2015**, *27*, 1738-1744.
93. Huebsch, N.; Lippens, E.; Lee, K.; Mehta, M.; Koshy, S.T.; Darnell, M.C.; Desai, R.; Madl, C.M.; Xu, M.; Zhao, X. Matrix elasticity of void-forming hydrogels controls transplanted stem cell-mediated bone formation. *Nat. Mater.* **2015**, *14*, 1269.
94. Sun, J.; Wei, D.; Zhu, Y.; Zhong, M.; Zuo, Y.; Fan, H.; Zhang, X. A spatial patternable macroporous hydrogel with cell-affinity domains to enhance cell spreading and differentiation. *Biomaterials* **2014**, *35*, 4759-4768.
95. Hennink, W.; Van Nostrum, C.F. Novel crosslinking methods to design hydrogels. *Adv. Drug Delivery Rev.* **2012**, *64*, 223-236.
96. Gacesa, P. Alginates. *Carbohydr. Polym.* **1988**, *8*, 161-182.
97. Goosen, M.F.; O'Shea, G.M.; Gharapetian, H.M.; Chou, S.; Sun, A.M. Optimization of microencapsulation parameters: Semipermeable microcapsules as a bioartificial pancreas. *Biotechnol. Bioeng.* **1985**, *27*, 146-150.
98. Gombotz, W.R.; Wee, S.F. Protein release from alginate matrices. *Adv. Drug Delivery Rev.* **2012**, *64*, 194-205.

99. Chenite, A.; Chaput, C.; Wang, D.; Combes, C.; Buschmann, M.; Hoemann, C.; Leroux, J.; Atkinson, B.; Binette, F.; Selmani, A. Novel injectable neutral solutions of chitosan form biodegradable gels in situ. *Biomaterials* **2000**, *21*, 2155-2161.
100. Azevedo, S.; Costa, A.; Andersen, A.; Choi, I.S.; Birkedal, H.; Mano, J.F. Bioinspired ultratough hydrogel with fast recovery, self‐healing, injectability and cytocompatibility. *Adv. Mater.* **2017**.
101. Waite, J.H.; Housley, T.J.; Tanzer, M.L. Peptide repeats in a mussel glue protein: Theme and variations. *Biochemistry* **1985**, *24*, 5010-5014.
102. Janes, K.A.; Fresneau, M.P.; Marazuela, A.; Fabra, A.; Alonso, M.a.J. Chitosan nanoparticles as delivery systems for doxorubicin. *J. Controlled Release* **2001**, *73*, 255-267.
103. Song, Y.; Lee, C.-S. In situ gelation of monodisperse alginate hydrogel in microfluidic channel based on mass transfer of calcium ions. *Korean Chemical Engineering Research* **2014**, *52*, 632-637.
104. Rowley, J.A.; Madlambayan, G.; Mooney, D.J. Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* **1999**, *20*, 45-53.
105. O'leary, L.E.; Fallas, J.A.; Bakota, E.L.; Kang, M.K.; Hartgerink, J.D. Multi-hierarchical self-assembly of a collagen mimetic peptide from triple helix to nanofibre and hydrogel. *Nature chemistry* **2011**, *3*, 821-828.
106. Zhang, W.; Wang, X.; Wang, S.; Zhao, J.; Xu, L.; Zhu, C.; Zeng, D.; Chen, J.; Zhang, Z.; Kaplan, D.L. The use of injectable sonication-induced silk hydrogel for VEGF 165 and BMP-2 delivery for elevation of the maxillary sinus floor. *Biomaterials* **2011**, *32*, 9415-9424.
107. Yokoyama, F.; Masada, I.; Shimamura, K.; Ikawa, T.; Monobe, K. Morphology and structure of highly elastic poly (vinyl alcohol) hydrogel prepared by repeated freezing-and-melting. *Colloid. Polym. Sci.* **1986**, *264*, 595-601.
108. Peppas, N.A.; Scott, J.E. Controlled release from poly (vinyl alcohol) gels prepared by freezing-thawing processes. *J. Controlled Release* **1992**, *18*, 95-100.
109. Akiyoshi, K.; Deguchi, S.; Moriguchi, N.; Yamaguchi, S.; Sunamoto, J. Self-aggregates of hydrophobized polysaccharides in water. Formation and characteristics of nanoparticles. *Macromolecules* **1993**, *26*, 3062-3068.
110. Qu, X.; Wirsén, A.; Albertsson, A.C. Structural change and swelling mechanism of pH‐sensitive hydrogels based on chitosan and D, L‐lactic acid. *J. Appl. Polym. Sci.* **1999**, *74*, 3186-3192.
111. Haglund, B.O.; Joshi, R.; Himmelstein, K.J. An in situ gelling system for parenteral delivery. *J. Controlled Release* **1996**, *41*, 229-235.
112. Djabourov, M.; Leblond, J.; Papon, P. Gelation of aqueous gelatin solutions. Ii. Rheology of the sol-gel transition. *J. Phys.* **1988**, *49*, 333-343.
113. Brigham, M.D.; Bick, A.; Lo, E.; Bendali, A.; Burdick, J.A.; Khademhosseini, A. Mechanically robust and bioadhesive collagen and photocrosslinkable hyaluronic acid semi-interpenetrating networks. *Tissue Eng. Part A* **2008**, *15*, 1645-1653.
114. Balakrishnan, B.; Joshi, N.; Banerjee, R. Borate aided schiff's base formation yields in situ gelling hydrogels for cartilage regeneration. *J Mater Chem B* **2013**, *1*, 5564-5577.
115. Cheaburu Yilmaz, C.N.; Pamfil, D.; Vasile, C.; Bibire, N.; Lupuşoru, R.-V.; Zamfir, C.-L.; Lupușoru, C.E. Toxicity, biocompatibility, pH-responsiveness and methotrexate release from

PVA/hyaluronic acid cryogels for psoriasis therapy. *Polymers* **2017**, *9*, 123.

116. Omobono, M.A.; Zhao, X.; Furlong, M.A.; Kwon, C.H.; Gill, T.J.; Randolph, M.A.; Redmond, R.W. Enhancing the stiffness of collagen hydrogels for delivery of encapsulated chondrocytes to articular lesions for cartilage regeneration. *J Biomed Mater Res A* **2015**, *103*, 1332-1338.
117. Park, Y.D.; Tirelli, N.; Hubbell, J.A. Photopolymerized hyaluronic acid-based hydrogels and interpenetrating networks. *Biomaterials* **2003**, *24*, 893-900.
118. Bryant, S.J.; Nuttelman, C.R.; Anseth, K.S. Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts in vitro. *Journal of Biomaterials Science, Polymer Edition* **2000**, *11*, 439-457.
119. Fedorovich, N.E.; Oudshoorn, M.H.; van Geemen, D.; Hennink, W.E.; Alblas, J.; Dhert, W.J. The effect of photopolymerization on stem cells embedded in hydrogels. *Biomaterials* **2009**, *30*, 344-353.
120. Bartnikowski, M.; Bartnikowski, N.; Woodruff, M.; Schrobback, K.; Klein, T. Protective effects of reactive functional groups on chondrocytes in photocrosslinkable hydrogel systems. *Acta Biomater.* **2015**, *27*, 66-76.
121. Browning, M.B.; Cosgriff-Hernandez, E. Development of a biostable replacement for PEGDA hydrogels. *Biomacromolecules* **2012**, *13*, 779-786.
122. Nettles, D.L.; Vail, T.P.; Morgan, M.T.; Grinstaff, M.W.; Setton, L.A. Photocrosslinkable hyaluronan as a scaffold for articular cartilage repair. *Ann. Biomed. Eng.* **2004**, *32*, 391-397.
123. Erickson, I.E.; Huang, A.H.; Sengupta, S.; Kestle, S.; Burdick, J.A.; Mauck, R.L. Macromer density influences mesenchymal stem cell chondrogenesis and maturation in photocrosslinked hyaluronic acid hydrogels. *Osteoarthritis and Cartilage* **2009**, *17*, 1639-1648.
124. Li, Q.; Williams, C.G.; Sun, D.D.; Wang, J.; Leong, K.; Elisseeff, J.H. Photocrosslinkable polysaccharides based on chondroitin sulfate. *J Biomed Mater Res A* **2004**, *68*, 28-33.
125. Amsden, B.G.; Sukarto, A.; Knight, D.K.; Shapka, S.N. Methacrylated glycol chitosan as a photopolymerizable biomaterial. *Biomacromolecules* **2007**, *8*, 3758-3766.
126. Yue, K.; Trujillo-de Santiago, G.; Alvarez, M.M.; Tamayol, A.; Annabi, N.; Khademhosseini, A. Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. *Biomaterials* **2015**, *73*, 254-271.
127. Nichol, J.W.; Koshy, S.T.; Bae, H.; Hwang, C.M.; Yamanlar, S.; Khademhosseini, A. Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials* **2010**, *31*, 5536-5544.
128. Williams, C.G.; Malik, A.N.; Kim, T.K.; Manson, P.N.; Elisseeff, J.H. Variable cytocompatibility of six cell lines with photoinitiators used for polymerizing hydrogels and cell encapsulation. *Biomaterials* **2005**, *26*, 1211-1218.
129. Fujisawa, S.; Atsumi, T.; Kadoma, Y.; Sakagami, H. Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity. *Toxicology* **2002**, *177*, 39-54.
130. Balakrishnan, B.; Banerjee, R. Biopolymer-based hydrogels for cartilage tissue engineering. *Chem. Rev.* **2011**, *111*, 4453-4474.
131. Yeh, J.; Ling, Y.; Karp, J.M.; Gantz, J.; Chandawarkar, A.; Eng, G.; Blumling Iii, J.; Langer, R.; Khademhosseini, A. Micromolding of shape-controlled, harvestable cell-laden hydrogels. *Biomaterials* **2006**, *27*, 5391-5398.

132. Loessner, D.; Meinert, C.; Kaemmerer, E.; Martine, L.; Yue, K.; Levett, P.A.; Klein, T.J.; Melchels, F.P.; Khademhosseini, A.; Hutmacher, D.W. Functionalization, preparation and use of cell-laden gelatin methacryloyl–based hydrogels as modular tissue culture platforms. *Nat. Protoc.* **2016**, *11*, 727-746.
133. Billiet, T.; Gasse, B.V.; Gevaert, E.; Cornelissen, M.; Martins, J.C.; Dubruel, P. Quantitative contrasts in the photopolymerization of acrylamide and methacrylamide‐functionalized gelatin hydrogel building blocks. *Macromol. Biosci.* **2013**, *13*, 1531-1545.
134. Terakado, M.; Yamazaki, M.; Tsujimoto, Y.; Kawashima, T.; Nagashima, K.; Ogawa, J.; Fujita, Y.; Sugiya, H.; Sakai, T.; Furuyama, S. Lipid peroxidation as a possible cause of benzoyl peroxide toxicity in rabbit dental pulp—a microsomal lipid peroxidation in vitro. *J. Dent. Res.* **1984**, *63*, 901-905.
135. Atsumi, T.; Murata, J.; Kamiyanagi, I.; Fujisawa, S.; Ueha, T. Cytotoxicity of photosensitizers camphorquinone and 9-fluorenone with visible light irradiation on a human submandibular-duct cell line in vitro. *Archives of oral biology* **1998**, *43*, 73-81.
136. Villanueva, I.; Weigel, C.A.; Bryant, S.J. Cell–matrix interactions and dynamic mechanical loading influence chondrocyte gene expression and bioactivity in PEG-RGD hydrogels. *Acta Biomater.* **2009**, *5*, 2832-2846.
137. Burdick, J.A.; Anseth, K.S. Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. *Biomaterials* **2002**, *23*, 4315-4323.
138. Hwang, N.S.; Varghese, S.; Zhang, Z.; Elisseeff, J. Chondrogenic differentiation of human embryonic stem cell–derived cells in arginine-glycine-aspartate–modified hydrogels. *Tissue Eng.* **2006**, *12*, 2695-2706.
139. Griffith, L.G.; Naughton, G. Tissue engineering--current challenges and expanding opportunities. *Science* **2002**, *295*, 1009-1014.
140. Chaudhuri, O.; Gu, L.; Klumpers, D.; Darnell, M.; Bencherif, S.A.; Weaver, J.C.; Huebsch, N.; Lee, H.-p.; Lippens, E.; Duda, G.N. Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat. Mater.* **2016**, *15*, 326-334.
141. Chaudhuri, O.; Gu, L.; Klumpers, D.; Darnell, M.; Bencherif, S.A.; Weaver, J.C.; Huebsch, N.; Lee, H.-p.; Lippens, E.; Duda, G.N. Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat. Mater.* **2016**, *15*, 326.
142. Bryant, S.J.; Anseth, K.S. Hydrogel properties influence ecm production by chondrocytes photoencapsulated in poly (ethylene glycol) hydrogels. *J Biomed Mater Res A* **2002**, *59*, 63-72.
143. Burdick, J.A.; Chung, C.; Jia, X.; Randolph, M.A.; Langer, R. Controlled degradation and mechanical behavior of photopolymerized hyaluronic acid networks. *Biomacromolecules* **2005**, *6*, 386-391.
144. Jeon, O.; Bouhadir, K.H.; Mansour, J.M.; Alsberg, E. Photocrosslinked alginate hydrogels with tunable biodegradation rates and mechanical properties. *Biomaterials* **2009**, *30*, 2724-2734.
145. Ruoslahti, E.; Pierschbacher, M.D. New perspectives in cell adhesion: RGD and integrins. *Science* **1987**, *238*, 491-498.
146. Steinmetz, N.J.; Bryant, S.J. The effects of intermittent dynamic loading on chondrogenic and osteogenic differentiation of human marrow stromal cells encapsulated in RGD-modified poly (ethylene glycol) hydrogels. *Acta Biomater.* **2011**, *7*, 3829-3840.

147. Schuh, E.; Hofmann, S.; Stok, K.; Notbohm, H.; Müller, R.; Rotter, N. Chondrocyte redifferentiation in 3D: The effect of adhesion site density and substrate elasticity. *J Biomed Mater Res A* **2012**, *100*, 38-47.
148. Knudson, C.B.; Knudson, W. Hyaluronan and CD44: Modulators of chondrocyte metabolism. *Clinical orthopaedics and related research* **2004**, *427*, S152-S162.
149. Hoffman, A.S. Hydrogels for biomedical applications. *Adv. Drug Delivery Rev.* **2012**, *64*, 18-23.
150. Murphy, C.M.; Haugh, M.G.; O'Brien, F.J. The effect of mean pore size on cell attachment, proliferation and migration in collagen–glycosaminoglycan scaffolds for bone tissue engineering. *Biomaterials* **2010**, *31*, 461-466.
151. Ko, Y.-G.; Kawazoe, N.; Tateishi, T.; Chen, G. Preparation of novel collagen sponges using an ice particulate template. *Journal of Bioactive and Compatible Polymers* **2010**, *25*, 360-373.
152. Zhang, J.; Yang, Z.; Li, C.; Dou, Y.; Li, Y.; Thote, T.; Wang, D.-a.; Ge, Z. Cells behave distinctly within sponges and hydrogels due to differences of internal structure. *Tissue Eng. Part A* **2013**, *19*, 2166-2175.
153. Zhang, J.; Wu, Y.; Thote, T.; Lee, E.H.; Ge, Z.; Yang, Z. The influence of scaffold microstructure on chondrogenic differentiation of mesenchymal stem cells. *Biomed. Mater.* **2014**, *9*, 035011.
154. Badiger, M.V.; McNeill, M.E.; Graham, N.B. Porogens in the preparation of microporous hydrogels based on poly (ethylene oxides). *Biomaterials* **1993**, *14*, 1059-1063.
155. Fu, J.; Fan, C.; Lai, W.S.; Wang, D. Enhancing vascularization of a gelatin-based micro-cavitary hydrogel by increasing the density of the micro-cavities. *Biomed. Mater.* **2016**, *11*, 055012.
156. Leong, W.; Lau, T.T.; Wang, D.-A. A temperature-cured dissolvable gelatin microsphere-based cell carrier for chondrocyte delivery in a hydrogel scaffolding system. *Acta Biomater.* **2013**, *9*, 6459-6467.
157. Ng, S.S.; Su, K.; Li, C.; Chan-Park, M.B.; Wang, D.-A.; Chan, V. Biomechanical study of the edge outgrowth phenomenon of encapsulated chondrocytic isogenous groups in the surface layer of hydrogel scaffolds for cartilage tissue engineering. *Acta Biomater.* **2012**, *8*, 244-252.
158. Kong, H.J.; Smith, M.K.; Mooney, D.J. Designing alginate hydrogels to maintain viability of immobilized cells. *Biomaterials* **2003**, *24*, 4023-4029.

Chapter 2

Preparation of gelatin methacrylate hydrogels with different stiffness for chondrocytes 3D culture

2.1 Summary

Gelatin hydrogels can mimic the microenvironments of nature tissue and encapsulate cells homogeneously, which makes them attractive for cartilage tissue engineering. Both the mechanical and biochemical properties of hydrogels can affect the phenotype of chondrocytes. However, the influence of each property on chondrocyte phenotype is unclear due to the difficulty to separate the roles of these properties. In this part, we aimed to study the influence of hydrogel stiffness on chondrocyte phenotype while excluding the role of biochemical factors such as adhesion site density in the hydrogels. By altering the degree of methacryloyl functionalization, gelatin hydrogels with different stiffness of 3.8, 17.1 and 29.9 kPa Young's modulus were prepared from the same concentration of gelatin methacryloyl (GelMA) macromers. Bovine articular chondrocytes were encapsulated in the hydrogels and cultured for 14 days. The influence of hydrogel stiffness on the cell behaviors including cell viability, cell morphology and maintenance of chondrogenic phenotype was evaluated. GelMA hydrogels with high stiffness (29.9 kPa) showed the best results on maintaining chondrogenic phenotype. These results will be useful for the design and preparation of scaffolds for cartilage tissue engineering.

2.2 Introduction

Cartilage defect is a common disease in our daily life, while the self-healing capacity of cartilage is very limited due to the lack of vasculature for nutrition supply. Therefore, articular cartilage repair using tissue engineering approach has drawn growing attention.[1] As we know, scaffolds, cells and growth factors are generally needed for tissue engineering. The interaction between cells and scaffolds plays an important role in controlling cell functions. Many factors can affect the cell-scaffold interaction, such as biological cues and

physical cues.[2,3] Biological cues are generally transmitted through the receptors on the cell membrane (e.g. integrin) and bioactive molecules in the extracellular matrices, which have been well studied.[4,5] The influence of physical cues, such as surface charges, topography and mechanical properties, on cell functions has also been extensively investigated.[6-9] As one of the physical cues, matrix stiffness has been reported to affect cell behaviors, such as cell spreading, migration, proliferation and differentiation.[10-13] Young's modulus of native tissue is different depending on tissue types.[14] Therefore, the matrix stiffness needs to be optimized for different cell types and the cellular response to matrix stiffness should be well studied. The fates of some types of cells, such as fibroblasts,[15] neutrophils,[16] and mesenchymal stem cells (MSCs),[17] have been found to be affected by matrix stiffness. For chondrocytes, it has been reported that substrates with a low stiffness can help to maintain their chondrogenic phenotype due to the round morphology of chondrocyte.[18] However, the result is based on 2D cell culture on hydrogel surface that cannot well mimic the 3D mechanical microenvironment in native cartilage. Therefore, it is necessary to study the optimal stiffness of matrix for maintaining chondrogenic phenotype in 3D culture platform.

A large variety of synthetic or natural materials have been used to fabricate hydrogels with controlled stiffness. For example, polyethylene glycol (PEG) hydrogels with Young's modulus from 34 kPa to 1370 kPa have been synthesized through photo-polymerizing by using different concentrations of macromer.[19] Arginine-glycine-aspartic acid (RGD) peptide incorporating polyethylene glycol dimethacrylate (PEGDM) hydrogels with Young's modulus from 2 kPa to 6 kPa have been used to study the chondrocyte extracellular matrix formation and phenotype maintenance.[20] Hydrogels of hyaluronic acid (HA),[21] agarose,[22] and alginate,[23] with gradient Young's modulus have also be prepared by altering crosslinking density or macromer concentration. Gelatin as a proteinaceous material is derived from collagen by hydrolytic degradation.[24,25] Gelatin has similar chemical composition to that of collagen, which makes gelatin to be one of the most useful biomaterials for tissue engineering.[26-28] Gelatin hydrogels have been used to investigate the interaction between matrix and cells because of their similarity to the in vivo microenvironments.[29,30]

To prepare gelatin hydrogels, photocrosslinking method has been proposed because of the advantages of injectability, mild cross-linking condition and low cytotoxicity.[31,32] Furthermore, the stiffness of photo-crosslinked gelatin hydrogels can be controlled by different degree of cross-linking. Introduction of methacryloyl group in gelatin molecules is a frequently used method to prepare photoreactive gelatin derivatives. Different strategies have been used to adjust the stiffness of gelatin methacryloyl (GelMA) hydrogels. It has been reported that the stiffness of GelMA hydrogels can be tethered from 5 kPa to 20 kPa by blending GelMA with other polymers, such as alginate and HA.[33] Variation of GelMA concentration can also result in different stiffness of GelMA hydrogels.[28,34] However, in these cases, it is difficult to separate the influence from stiffness and other factors, such as composition and concentration. Therefore, it is desirable to prepare hydrogels with different stiffness while keeping their composition and matrix concentration at the same level. Such hydrogels may separate the influence of stiffness from that of other factors, such as composition, matrix concentration and adhesion site density.

In this study, the influence of matrix stiffness on the maintenance of chondrocyte phenotype was investigated by using a series of GelMA hydrogels that had different stiffness but the same gelatin concentration. The GelMA hydrogels were prepared by adjusting the degree of GelMA functionalization. Bovine articular chondrocytes were encapsulated in these hydrogels through photocrosslinking method and then cultured for 14 days. The behaviors and fates of chondrocytes in the hydrogels were studied by using cytoskeleton staining, ECM secretion assay, histological staining and gene expression.

2.3 Materials and methods

2.3.1 Synthesis of GelMA macromers

GelMA macromers were synthesized according to a previously described method.[35] 5 g gelatin (type A, 300 bloom, Sigma-Aldrich, MO, USA) was dissolved in 45 mL of phosphate buffered saline (PBS) at 60 ^{o}C under stirring to obtain a 10 wt% aqueous gelatin solution. For the synthesis of GelMA macromer with high degree of functionalization (DoF), the pH of buffer solution was adjusted to 7.6 with 1N sodium hydroxide solution (Wako, Osaka, Japan) before gelatin dissolution. Different volume of methacrylic anhydride (MA, Sigma-Aldrich, MO, USA) (0.2 mL, 1 mL and 5 mL) was added into the gelatin solution at a rate of 0.5 mL/minutes under stirring at 50 ^{o}C to prepare GelMA with different introduction ratio of MA. After reaction in dark for 3 hours, the products were diluted with 5-fold warm PBS (50 ^{o}C) and then dialyzed against Milli-Q water for 7 days at 40 ^{o}C using a dialysis membrane (12-14 kD molecular weight cut-off, Spectrum Laboratories Inc. CA, USA) to remove salts and excess free MA. After that, the products were lyophilized for 2 days to obtain white porous foam and stored at -20 ^{o}C for further use.

2.3.2 ^{1}H nuclear magnetic resonance (NMR)

The degree of methacryloyl functionalization was quantified by using ^{1}H NMR according to a previously described method.[36] ^{1}H NMR spectra were collected by using an NMR spectrometer (AL300; JEOL, Tokyo, Japan) with a single axis gradient inverse probe at a frequency of 300 MHz. Before the measurement, 20 mg of GelMA macromers was completely dissolved in 1 mL deuterium oxide (containing 0.05 wt% 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt for calibration, Sigma-Aldrich, MO, USA). The gelatin without functionalization was also examined for calculating the degree of methacryloyl substitution using the following equation (1).

DoF = 1- (lysine methylene proton of GelMA)/(lysine methylene proton of Gelatin) ×100% (1)

2.3.3 RGD density measurement

The RGD density in GelMA macromers was quantified by reacting arginine groups with 9, 10-phenanthrenequinone (Wako, Osaka, Japan) to produce a fluorescence compound. Briefly, 1 mg/mL gelatin or GelMA was mixed with 300 μL of an ethanol solution of 9, 10-phenanthrenequinone (150 μM) and 50 μL of NaOH aqueous solution (2 N). The mixture was incubated at 60 °C under dark for 3 hours. Then 200 μL of each sample was mixed with 200 μL of HCl (1.2 N) and the mixture was allowed to stand at room temperature under dark for 1 hour. The emission of the mixture was measured at an excitation wavelength of 312 nm by an FP-6500 spectrofluorometer (JASCO, Tokyo, Japan).[37,38]

2.3.4 Preparation of GelMA hydrogels

GelMA macromers (10 wt%) and photo-initiator, 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (Irgacure 2959, Sigma-Aldrich, USA) (5 mg/mL), were dissolved in PBS at 50 ^{o}C. The solution was added to the space between two quartz glass coverslips separated by a 1 mm-thick

spacer made from silicone gel sheet (KOKEN Co., Ltd., Tokyo, Japan). The construct was exposed to 365 nm UV light (CL-1000, Funakoshi Co., Ltd., Japan) at a distance of 20 cm for 2 minutes.

2.3.5 Mechanical testing

Atomic force microscopy (AFM, MFP-3D-Bio) was applied to determine the stiffness of GelMA hydrogels. The hydrogel samples were incubated in PBS at 37 ^{o}C for 24 hours and then their stiffness was measured at room temperature. An optical microscope was used to control the position of the AFM tip. Silicon nitride cantilevers (Bruker, CA, USA) with a 600 nm diameter glass ball were used as the probe. The exact spring constant was measured before each experiment using a thermal tuning method. The force curves were collected and fitted to Hertz's contact model to calculate Young's modulus.[8] Three samples of each group were measured for calculation of means and standard deviations.

2.3.6 Swelling ratio measurement

To determine the mass swelling ratio, hydrogels were punched into disks with a 6 mm biopsy punch. The hydrogel disks were soaked in PBS at 37 ^{o}C for 24 hours. The samples were blotted with a KimWipe paper to remove the residual liquid and weighed to obtain the equilibrium wet weight. The dry weight was the sample weight after freeze-drying. The mass swelling ratio was calculated by dividing the equilibrium wet weight by the dry weight of the hydrogel disks.[34]

2.3.7 Measurement of enzymatic degradation of hydrogels

GelMA hydrogels with different stiffness were prepared and punched into disks with an 8 mm diameter biopsy punch following by swollen in PBS for 24 hours to reach swelling equilibrium. The swollen hydrogel disks were immersed in 2 ml of PBS containing 10 units mL^{-1} of collagenase (Worthington Biochemical, Lakewood, USA) and incubated at 37 ^{o}C in an orbital shaker at a shaking speed of 60 rpm. At the time points of 1, 2, 4, 9 and 20 hours, the hydrogel samples were taken out and weighed after being blotted. The degradation degree of the hydrogels was determined by normalizing the residual hydrogel wet weight to the initial wet weight. Three samples were used at every time point for the measurement to calculate means and standard deviations.

2.3.8 Culture of chondrocytes in hydrogels

Chondrocytes were isolated from articular cartilage from the knees of a 9 week-old calf according to previously reported protocol.[39,40] The isolated primary chondrocytes were cultured in 75 cm^2 tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 4500 mg L^{-1} glucose, 4 mM glutamine, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 1 mM sodium pyruvate and 50 µg mL^{-1} ascorbic acid at 37 ^{o}C and 5% CO_2. The cells were subcultured after confluence and the cell culture medium was refreshed every 3 days. The chondrocytes at passage 2 were used in the following experiments. The cells were detached with a trypsin/EDTA solution, collected by centrifugation, counted with a hemocytometer and resuspended in the PBS solutions containing GelMA macromers (111 mg/mL) and photo-initiator Irgacure 2959 (5 mg/mL) to

obtain cell suspension at a density of 2×10^7 cell/mL. The suspension solution was used to prepare cell-laden hydrogels in the same manner as above described. The quartz glass coverslips were sterilized before usage and all the experimental procedures were conducted sterilely in a clean bench. The cell-laden hydrogels were then punched into disks (6 mm diameter × 1 mm thickness) with a sterile 6 mm biopsy punch. The cells in the hydrogel disks were cultured in DMEM medium at 37 ºC and 5% CO_2 under shaking for 14 days and the medium was changed every two days.

2.3.9 Cell viability assay

Live/dead staining was performed to evaluate cell viability of chondrocytes in the hydrogels by using Cellstain Double Staining Kit (Dojindo Laboratories, Tokyo, Japan). After 1 and 7 days of culture, the cell-laden hydrogel disks were washed with PBS and incubated with serum-free medium containing calcein-AM (2 μM) and propidium iodide (4 μM) for 15 minutes. After that, the live cells and dead cells in the interior areas of hydrogels were observed under a fluorescence microscope.

2.3.10 Cytoskeleton actin filament staining

The cytoskeleton of chondrocytes in the hydrogels after 7 and 14 days of culture was observed by actin filament (F-actin) staining. The cell-laden hydrogel disks were washed with PBS for 3 times, fixed with 4 % paraformaldehyde at 4 ºC for 24 hours and further washed 2 times with PBS. Then the cell-laden hydrogel disks were immersed in 5 mL of 0.2% Triton X-100 for 50 minutes to permeabilize the cells. After being washed with PBS for 3 times and blocked with 1% bovine serum albumin (BSA) solution at room temperature for 30 minutes, the samples were immersed in 1 mL PBS containing 40-fold diluted Alexa FluorVR 488 phalloidin (Invitrogen, Carlsbad, CA) for 60 minutes to stain actin filaments. Cell nuclei were stained with 1000-fold diluted 4’, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Dojindo Laboratories, Tokyo, Japan) solution in PBS at room temperature for 10 minutes. After staining, cell-laden hydrogel disks were rinsed with PBS again and their fluorescence images were captured using a confocal microscope (Zeiss LSM 510 Meta, Oberkochen, Germany).

2.3.11 Histological stainings

After 14 days of culture, the cell-laden hydrogel constructs were washed with PBS and fixed with 10% neutral buffer formalin at room temperature for 2 days. After that, the samples were dehydrated in a series of ethanol solution with increasing ethanol concentration from 70% to 99.5%, embedded in paraffin and sectioned to obtain cross-sections having 5 μm thickness. The cross-sections were deparaffinized and stained with hematoxylin and eosin dyes (HE staining) for cell morphology and safranin-O dye for glycosaminoglycan. The stained samples were observed under an optical microscope.

2.3.12 Quantification of DNA and sulfated glycosaminoglycan (sGAG)

DNA amount in each hydrogel was quantified to evaluate the proliferation of chondrocytes. The cell-laden hydrogels with different stiffness were harvested and freeze-dried after being cultured for 14 days. The freeze-dried cell-laden constructs were digested by 500 μL papain solution (Sigma-Aldrich, MO, USA)

prepared by dissolving papain at a concentration of 400 mg mL^{-1} in 0.1 M phosphate buffer (pH 6.0) containing 5 mM cysteine hydrochloride and 5 mM ethylenediaminetetraacetic acid (EDTA). 10 μL of papain digestion solution was used to measure the DNA content by using Hoechst 33258 dye (Sigma-Aldrich, MO, USA) to produce fluorescence. The fluorescence intensity was read with an FP-6500 spectrofluorometer (JASCO, Tokyo, Japan) at an excitation/emission wavelength of 360 nm and 460 nm. The sGAG content in each digestion solution was measured by using BlyscanTM Glycosaminoglycan Assay Kit (Biocolor, County Antrim, UK) according to the instruction offered by the product. Three samples in each group were used for the measurement to calculate means and standard deviations.

2.3.13 Real-time PCR analysis

Expression of genes encoding collagen type II and aggrecan in the chondrocytes cultured in the different stiffness hydrogels was analyzed by a real-time polymerase chain reaction (real-time PCR).[41] After being cultured for 14 days, the cell-laden hydrogel samples were washed with PBS for 3 times, frozen in liquid nitrogen and crushed into powder by an electric crusher. The powder samples were dissolved in Sepasol solution (1 mL per sample) to isolate the contained RNA. The RNA was converted to cDNA by MuLV reverse transcriptase (Applied Biosystems, Massachusetts, USA). Real-time PCR was used to amplify glyceraldehyde-3-phosphate dehydrogenase (Gapdh), type II collagen (Col2a1) and aggrecan (Acan). The reactions were run for 40 cycles using a 7500 real-time PCR System (Applied Belgium, USA). The expression level of Gapdh, a housekeeping gene, was used as an endogenous control. The expression level of the target gene was then calculated using the $2^{-\Delta\Delta Ct}$ formula with reference to the chondrocytes from 2D expansion culture. The primer and probe sequences were chosen according to a previous study. Three samples in each group were used for the measurement to calculate means and standard deviations.

2.3.14 Statistical analysis

All data were reported as the mean ± standard deviation (SD). Statistical analysis was performed using a one-way analysis of variance and Tukey's post hoc test for multiple comparisons. All statistical analyses were executed using KyPlot 2.0 beta 15.

2.4 Results and discussion

2.4.1 Synthesis and characterization of GelMA macromers

The GelMA macromers with three different degrees of functionalization were synthesized by adjusting the volume of methacrylic anhydride (0.2 mL, 1.0 mL and 5.0 mL per 5 g gelatin) during the reaction. The conjugation of methacryloyl groups to gelatin molecules was confirmed by the ^{1}H NMR spectra (Figure 2.1). The increase of signal at δ = 5.4 and 5.7 ppm (the proton of methacrylate vinyl group of MA) and a decrease of the signal at δ = 2.9 ppm (the protons of methylene of lysine signal) confirmed the increase of modification degree with the feed MA amount. Because the proton signal of the aromatic amino acid moieties in gelatin remained constant, their intensity was used to normalize the intensity of other protons in different samples. Therefore, the DoF was calculated by comparing the proton signal at δ = 2.9 ppm of

unmodified gelatin and GelMA. The DoF of the three types of GelMA macromers increased from 25.8 ± 0.7 % to 91.7 ± 1.4 % by adjusting the feed ratio of MA to gelatin (Table 2.1).

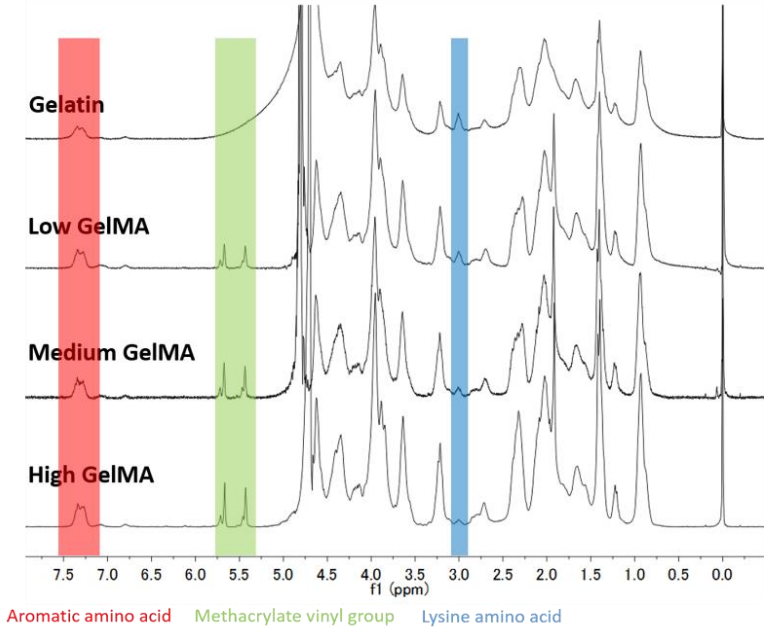

Figure 2.1 ^{1}H NMR spectra of unmodified gelatin (Gel), GelMA macromers with different DoF (Low GelMA, Medium GelMA and High GelMA).

Table 2.1 The DoF of Low GelMA, Medium GelMA and High GelMA

Sample	Feed ratio of Gel (g)/MA (mL)	DoF (%)
Low GelMA	5 / 0.2	25.8 ± 0.7
Medium GelMA	5 / 1.0	52.5 ± 1.2
High GelMA	5 / 5.0	91.7 ± 1.4

2.4.2 RGD density in gelatin and GelMA macromers

The arginine residues in gelatin also have amino groups that may react with MA. Therefore the content of arginine residues in the GelMA macromers was measured to confirm if the RGD density was changed after the modifications. The amino groups in arginine residues can react with 9, 10-phenanthrenequinone to produce a fluorescence compound. Unmodified gelatin and the three types of GelMA macromers with low, medium and high DoF had almost the same level of fluorescence absorbance (Figure 2.2). Blank control without RGD prepared by deionized water did not show obvious fluorescence absorbance at the wavelength range. The results indicated that the arginine content in Gel and GelMA macromers was almost the same, which suggests that the arginine should be not involved in the reaction even at a high DoF. Therefore the RGD density in all the GelMA hydrogels prepared from the three types of GelMA macromers should be kept at the same levels, which was important to exclude the influence of different density of active RGD motifs.

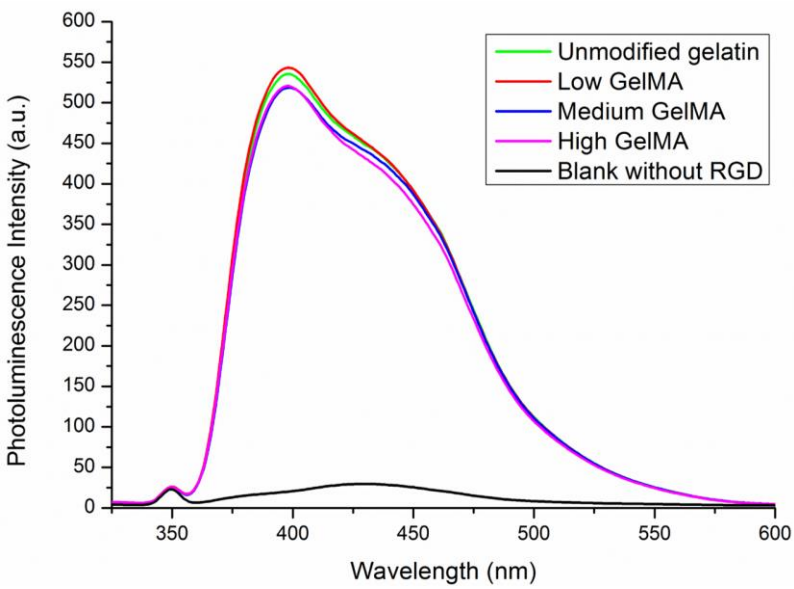

Figure 2.2 Photoluminescence spectra of 9, 10-phenanthrenequinone after reaction with gelatin and GelMA macromers.

2.4.3 Young's modulus and swelling ratio of GelMA hydrogels

Three types of hydrogels were prepared from GelMA macromers with the same concentration of gelatin (10 wt%) but different DoF. The stiffness of GelMA hydrogels was measured by AFM. The GelMA hydrogels prepared from GelMA with low, medium and high DoF had Young's modulus of 3.8 ± 0.3, 17.1 ± 2.4 and 29.9 ± 3.4 kPa, respectively (Figure 2.3a). As expected, high DoF resulted in GelMA hydrogels with significantly increased Young's modulus. The results showed that the GelMA hydrogels with a broad range of stiffness could be generated by adjusting the DoF of macromers.

Swelling ratio of hydrogels is an important factor that should be considered for tissue engineering application. The swelling ratio of GelMA hydrogels with low, medium and high stiffness was calculated to be 17.89 ± 1.24, 8.86 ± 0.20 and 7.20 ± 0.12, respectively (Figure 2.3b). Clearly, the swelling ratio of hydrogels decreased with the increase of stiffness. This may be because the high DoF of methacryloyl in high stiffness GelMA hydrogel could increase the density of cross-linking network. The high cross-linking density could restrain the swelling of the hydrogels and result in a low swelling ratio.

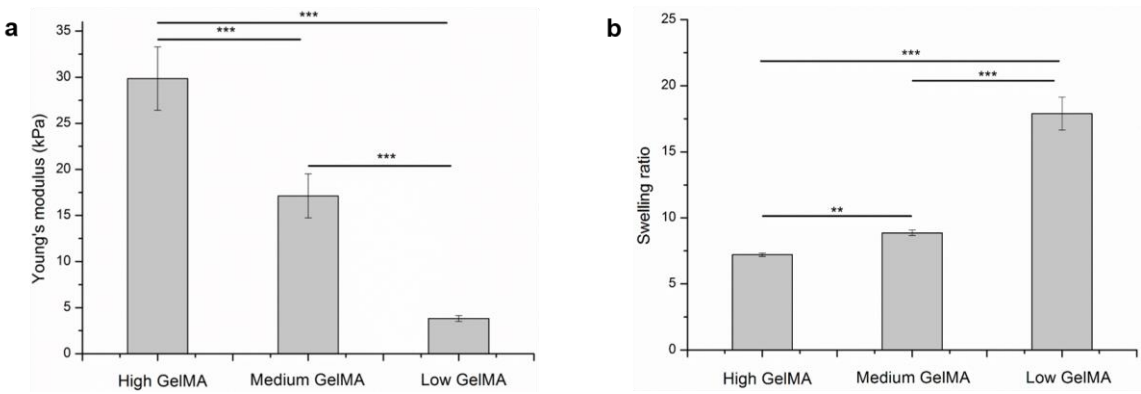

Figure 2.3 Young's modulus (a) and equilibrium swelling ratio (b) of GelMA hydrogels with high, medium and low stiffness. Means ± SD, N=3. ***, P < 0.001; **, P < 0.01.

2.4.4 Enzymatic degradation of GelMA hydrogels

It is necessary to investigate the biodegradability of hydrogels before their biomedical applications. The enzymatic degradability of GelMA hydrogels with different stiffness was examined in the presence of collagenase. Collagenase is a member of the matrix metalloproteinase (MMP) family that can degrade and remodel the extracellular matrix for cell spreading and migration.[42] Herein, collagenase was used because it can accelerate the degradation of proteolysis-sensitive gelatin hydrogels. The weight loss rate of low, medium and high stiffness GelMA hydrogels after treatments with collagenase is shown in Figure 2.4 GelMA hydrogels with higher stiffness were degraded more slowly when compared to those with lower stiffness. These results suggested that the enzymatic degradability of GelMA hydrogels could be controlled by the degree of cross-linking and hydrogel stiffness. Higher cross-linking degree resulted in higher stiffness and slower degradation.

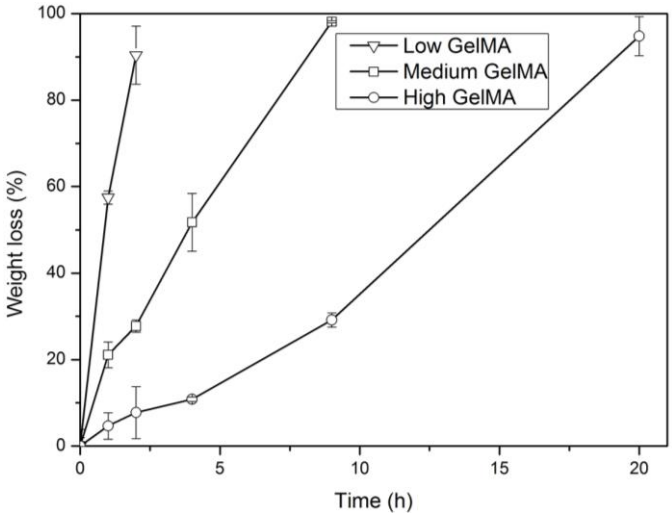

Figure 2.4 Enzymatic degradation of GelMA hydrogels in the presence of 10 unit mL^{-1} of collagenase at 37 ^{o}C. Means ± SD, N=3.

2.4.5 Cell viability in GelMA hydrogels

Cell viability was investigated by live/dead staining after chondrocytes were encapsulated in the GelMA hydrogels and cultured for 1 and 7 days. In all the groups, most of the chondrocytes were alive (green color) and only a few dead cells were observed (red color) after 1 and 7 days of culture (Figure 2.5). The results indicated that the cells in the hydrogels had high cell viability even after the photocrosslinking process, suggesting that it may be a good strategy to use the GelMA hydrogels for cell encapsulation. Furthermore, the live cell density seemed to be the same for all the GelMA hydrogels, suggesting that the stiffness of GelMA did not have an obvious influence on cell viability.

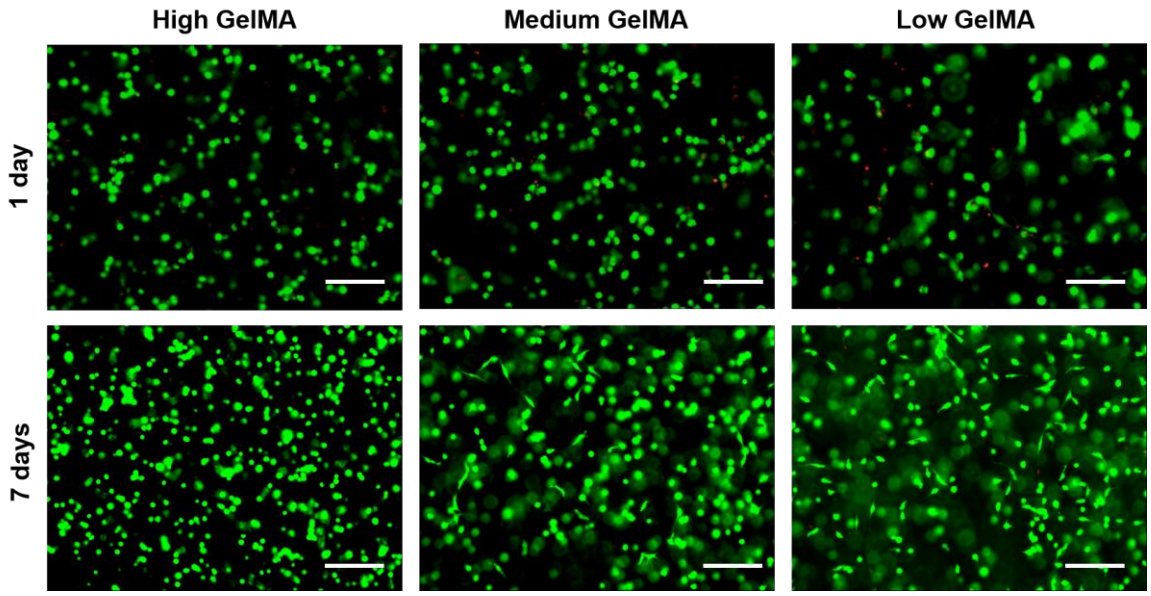

Figure 2.5 Live/dead staining of chondrocytes cultured in the GelMA hydrogels for 1 day and 7 days. Scale bar = 100 µm. (Green: live cells; Red: dead cells)

2.4.6 Cytoskeleton organization in GelMA hydrogels

Immunofluorescence staining of F-actin was performed to visualize the cellular morphology and cytoskeleton organization in the hydrogels (Figure 2.6). Chondrocytes in the high stiffness GelMA hydrogels had almost round shape and no obvious F-actin stretch, which indicated that the cell did not spread well after being cultured in the high stiffness hydrogels for 7 and 14 days. Furthermore, some cell clusters were observed in the high stiffness GelMA hydrogels. In contrast, the cell elongated in the low stiffness GelMA hydrogels with abundant F-actin filaments after culture for 7 and 14 days. Chondrocytes cultured in the medium stiffness GelMA hydrogels showed round morphology after 7 days of culture and spreading morphology after 14 days of culture. The results indicated that cell morphology could be controlled by the stiffness of hydrogels.

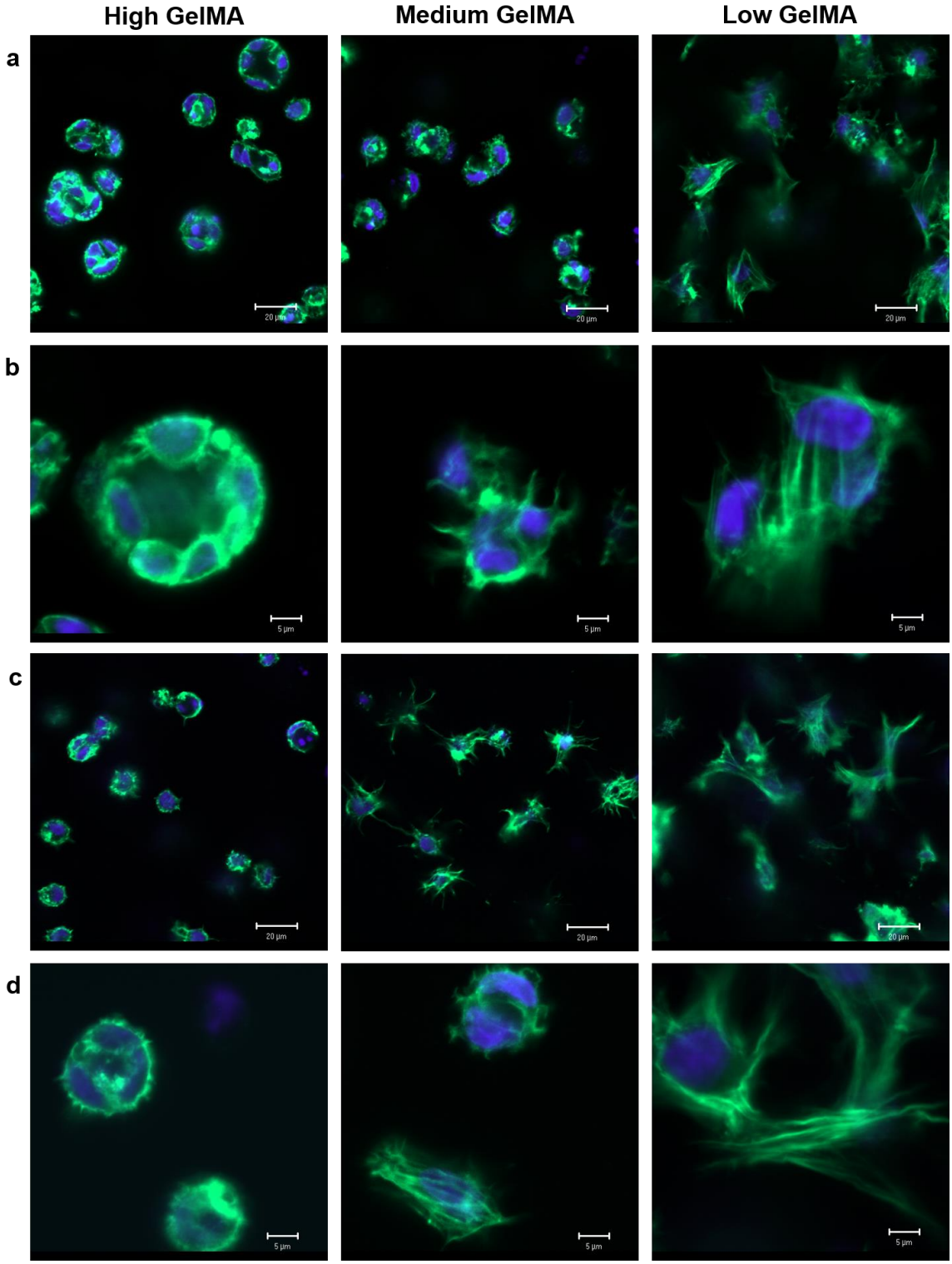

Figure 2.6 Confocal laser microscopy images of chondrocytes that were stained with F-actin (green) and cell nuclei (blue) after being cultured in GelMA hydrogels for 7 days (a, b) and 14 days (c, d). (a, c) scale bar = 20 µm; (b, d) scale bar = 5 µm.

Cytoskeletal configuration and regulation have been reported to play an important role in the process of chondrogenesis.[43] It was recognized that round cell morphology and decreased actin cytoskeletal

organization are beneficial for the chondrogenesis of mesenchymal stem cells (MSCs).[43,44] Spherical aggregation was also beneficial for chondrogenesis due to the increased intercellular contacts.[45,46] The high stiffness GelMA hydrogels had a higher Young's modulus and a higher degree of cross-linking, which inhibited cell spreading and resulted in weak F-actin filament network formation. The microenvironment of high stiffness hydrogel also promoted spherical aggregation. Similar cell morphology was observed in medium stiffness GelMA hydrogels after 7 days of culture. However, most cells in the medium stiffness GelMA hydrogels spread after 14 days of culture, which might be due to the continuous proteolytic degradation of hydrogels. This suggested the hydrogels with high stiffness could support the maintenance of the round morphology of chondrocytes, in agreement with the previous study.[47]

2.4.7 Histological stainings

Cell morphology and cartilaginous matrices production of the chondrocytes after 14 days of culture in the GelMA hydrogels were further investigated by histological staining (Figure 2.7). HE staining indicated a homogeneous cell distribution for all the GelMA hydrogels. The chondrocytes showed round morphology in the high stiffness GelMA hydrogels, while partially spreading morphology in medium stiffness GelMA hydrogels. In contrast, cells in the low stiffness GelMA hydrogels showed an elongated morphology. The elongation of cells in low stiffness hydrogels might be a signal of losing chondrogenic phenotype.[43,44] Safranin-O staining indicated that more proteoglycans were detected in the high stiffness GelMA hydrogels than the other two groups. The cells cultured in medium stiffness GelMA hydrogels showed partially positive staining of proteoglycans, while the chondrocytes cultured in the low stiffness GelMA hydrogels lost their proteoglycans secretion capacity obviously.

One reason for these results may be that high stiffness offered a better stimulus to chondrocyte through the pathways to maintain their chondrogenic phenotype in 3D culture.[44,48,49] Another reason may be that the GelMA hydrogels with high stiffness could promote the chondrocytes cluster formation. The chondrocytes aggregation has been reported to facilitate the outcome of cartilage repair,[50,51] because cell-cell interaction plays a critical role in chondrogenesis.[52-54] The staining results indicated that GelMA hydrogels with high stiffness could support the chondrogenic phenotype.

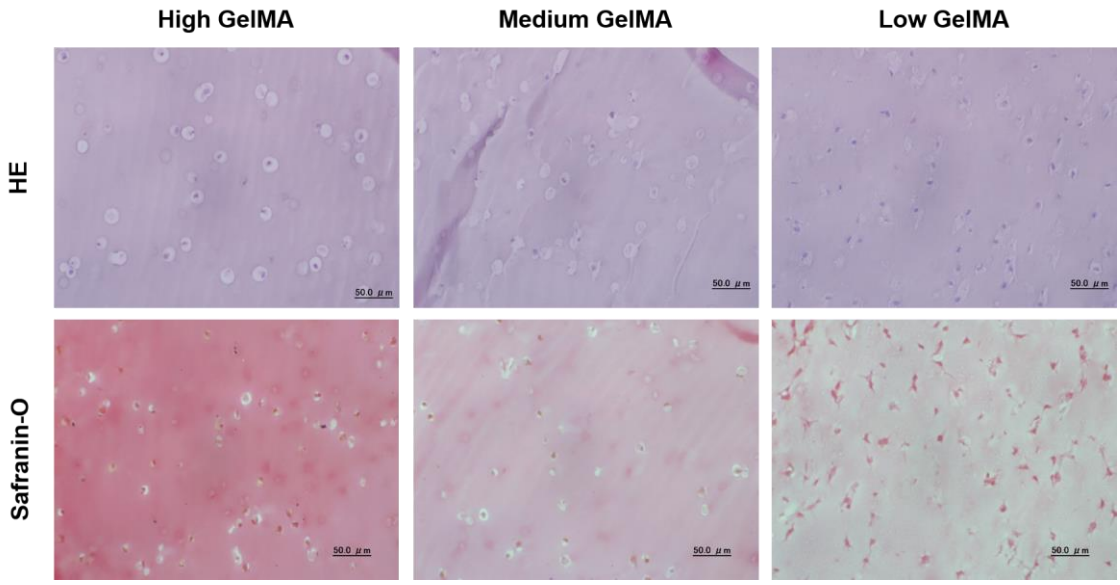

Figure 2.7 HE and safranin-O stainings of chondrocytes cultured in the GelMA hydrogels for 14 days.

2.4.8 Quantification of DNA, cartilaginous matrices and gene expression

To investigate whether the stiffness affects cell proliferation, DNA amount measurement was performed after the cells were cultured in the GelMA hydrogels for 14 days (Figure 2.8a). The cells cultured in different GelMA hydrogels showed almost the same DNA amount, indicating the stiffness of hydrogels had no obvious influence on cell proliferation.

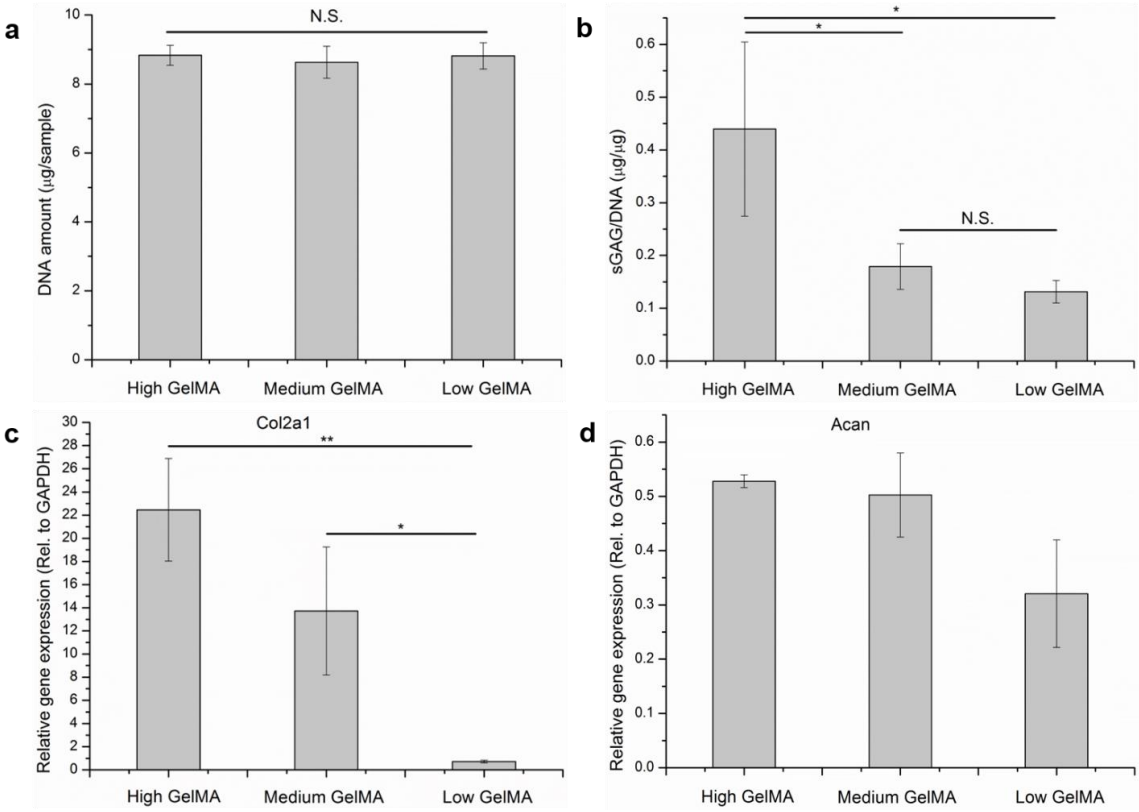

Figure 2.8 DNA amount (a), sGAG/DNA ratio (b) and expression of genes encoding collagen type II (c) and aggrecan (d) of chondrocytes cultured in the GelMA hydrogels for 14 days. Means ± SD, N=3. **, $P < 0.01$; *, $P < 0.05$.

The secretion of ECM proteins is very important for cartilage regeneration because chondrocytes in cartilage are surrounded by abundant ECMs, such as sGAG. The sGAG/DNA ratio was calculated from the amount of sGAG and DNA to show the production capacity of cartilaginous ECM by each cell (Figure 2.8b). The sGAG/DNA ratio of chondrocytes in high stiffness GelMA hydrogels was significantly higher than the other groups after 14 days of culture. No significant difference could be seen in the medium and low stiffness GelMA hydrogels.

The expression of genes encoding the two typical cartilaginous matrices (collagen type II and aggrecan) was analyzed by real-time PCR (Figure 2.8c, 2.8d). Chondrocytes cultured in high stiffness GelMA hydrogels showed the highest expression level of collagen type II and aggrecan genes after 14 days of culture. The quantification data of cartilaginous matrices and their gene expression were in good agreement with the staining results, indicating that chondrocytes cultured in the high stiffness GelMA hydrogels had the best maintenance capacity of chondrogenic phenotype.

The rapid degradation and stress relaxation characters of low stiffness GelMA hydrogels may promote the dedifferentiation of chondrocytes as they down-regulated the gene expression of collagen type II and

aggrecan. It has been reported that high spreading can be observed in physiological extracellular matrices with high degradation rate and quick stress relaxation.[55,56] All of our results suggested the GelMA hydrogels with high stiffness had the best function for the maintenance of chondrocyte phenotype.

The results of this study are different from some previous studies.[19,20,22] Some previous studies have reported that lower stiffness hydrogels had a better maintenance function of chondrogenic phenotype. The difference of stiffness range and material properties between this study and the previous studies might be the reasons for the different results. The high stiffness in this study was at the same range as the low stiffness of the previous studies. The Young's modulus of hydrogels in this study was controlled to mimic the mechanical property of cartilage ECM.[57-59] However, the stiffness range was far from the rigidity of native cartilage (400-800 kPa).[59,60] This might be one reason. The materials property including adhesion sites and degradable sites might be another reason. In PEG, agarose and HA hydrogels, cells keep round shape in lower stiffness hydrogels until sufficient extracellular matrices are produced.[61,62] But in gelatin-based hydrogels, cells can easily spread.[55] The presence of RGD in the hydrogels can promote cell spreading. It is important to investigate the influence of matrix stiffness on chondrogenic phenotype by using natural ECM protein materials (collagen and gelatin etc.) because they have RGD and MMP degradable sequences to better mimic the microenvironment as compared with other natural polymer or synthetic materials, such as HA and PEG.

2.5 Conclusions

GelMA hydrogels with different stiffness and same RGD density were fabricated by adjusting the degree of methacryloyl substitution of same concentration of gelatin. The Young's modulus increased with the degree of functionalization. Cell cultured in the hydrogels showed high viability. Bovine chondrocytes cultured in the high stiffness GelMA hydrogels showed rounder morphology, secreted more cartilaginous matrices and exhibited higher expression of collagen II and aggrecan genes compared to the cells cultured in medium and low stiffness GelMA hydrogels. The high-stiffness GelMA hydrogels had the highest capacity on maintaining the phenotype of chondrocytes.

2.6 References

1. Jeng, L.; Hsu, H.-P.; Spector, M. Tissue-engineered cartilaginous constructs for the treatment of caprine cartilage defects, including distribution of laminin and type IV collagen. *Tissue Eng. Part A* **2013**, *19*, 2267-2274.
2. Wang, X.; Song, W.; Kawazoe, N.; Chen, G. Influence of cell protrusion and spreading on adipogenic differentiation of mesenchymal stem cells on micropatterned surfaces. *Soft Matter* **2013**, *9*, 4160-4166.
3. Kumar, S.S.; Hsiao, J.-H.; Ling, Q.-D.; Dulinska-Molak, I.; Chen, G.; Chang, Y.; Chang, Y.; Chen, Y.H.; Chen, D.-C.; Hsu, S.-T. The combined influence of substrate elasticity and surface-grafted molecules on the ex vivo expansion of hematopoietic stem and progenitor cells. *Biomaterials* **2013**, *34*, 7632-7644.
4. Hoshiba, T.; Kawazoe, N.; Tateishi, T.; Chen, G. Development of extracellular matrices mimicking stepwise adipogenesis of mesenchymal stem cells. *Adv. Mater.* **2010**, *22*, 3042-3047.

5. Lu, H.; Kawazoe, N.; Kitajima, T.; Myoken, Y.; Tomita, M.; Umezawa, A.; Chen, G.; Ito, Y. Spatial immobilization of bone morphogenetic protein-4 in a collagen-PLGA hybrid scaffold for enhanced osteoinductivity. *Biomaterials* **2012**, *33*, 6140-6146.
6. Leung, M.; Jana, S.; Tsao, C.-T.; Zhang, M. Tenogenic differentiation of human bone marrow stem cells via a combinatory effect of aligned chitosan–poly-caprolactone nanofibers and TGF-β3. *J. Mater. Chem. B* **2013**, *1*, 6516-6524.
7. Guo, L.; Kawazoe, N.; Hoshiba, T.; Tateishi, T.; Chen, G.; Zhang, X. Osteogenic differentiation of human mesenchymal stem cells on chargeable polymer‐modified surfaces. *J. Biomed. Mater. Res. A* **2008**, *87*, 903-912.
8. Wang, X.; Nakamoto, T.; Dulińska-Molak, I.; Kawazoe, N.; Chen, G. Regulating the stemness of mesenchymal stem cells by tuning micropattern features. *J Mater Chem B* **2016**, *4*, 37-45.
9. Higuchi, A.; Ling, Q.-D.; Kumar, S.S.; Chang, Y.; Alarfaj, A.A.; Munusamy, M.A.; Murugan, K.; Hsu, S.-T.; Umezawa, A. Physical cues of cell culture materials lead the direction of differentiation lineages of pluripotent stem cells. *J Mater Chem B* **2015**, *3*, 8032-8058.
10. Sunyer, R.; Jin, A.J.; Nossal, R.; Sackett, D.L. Fabrication of hydrogels with steep stiffness gradients for studying cell mechanical response. *PLoS ONE* **2013,** *7*, e46107.
11. Friedl, P.; Wolf, K. Plasticity of cell migration: A multiscale tuning model. *J. cell Biol.* **2010**, *188*, 11-19.
12. Higuchi, A.; Ling, Q.-D.; Chang, Y.; Hsu, S.-T.; Umezawa, A. Physical cues of biomaterials guide stem cell differentiation fate. *Chem. Rev.* **2013**, *113*, 3297-3328.
13. Engler, A.J.; Sen, S.; Sweeney, H.L.; Discher, D.E. Matrix elasticity directs stem cell lineage specification. *Cell* **2006**, *126*, 677-689.
14. Levental, I.; Georges, P.C.; Janmey, P.A. Soft biological materials and their impact on cell function. *Soft Matter* **2007**, *3*, 299-306.
15. da Cunha, C.B.; Klumpers, D.D.; Li, W.A.; Koshy, S.T.; Weaver, J.C.; Chaudhuri, O.; Granja, P.L.; Mooney, D.J. Influence of the stiffness of three-dimensional alginate/collagen-I interpenetrating networks on fibroblast biology. *Biomaterials* **2014**, *35*, 8927-8936.
16. Yeung, T.; Georges, P.C.; Flanagan, L.A.; Marg, B.; Ortiz, M.; Funaki, M.; Zahir, N.; Ming, W.; Weaver, V.; Janmey, P.A. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil.Cytoskelet.* **2005**, *60*, 24-34.
17. Discher, D.E.; Janmey, P.; Wang, Y.-l. Tissue cells feel and respond to the stiffness of their substrate. *Science* **2005**, *310*, 1139-1143.
18. Schuh, E.; Kramer, J.; Rohwedel, J.; Notbohm, H.; Müller, R.; Gutsmann, T.; Rotter, N. Effect of matrix elasticity on the maintenance of the chondrogenic phenotype. *Tissue Eng. Part A* **2010**, *16*, 1281-1290.
19. Bryant, S.J.; Anseth, K.S. Hydrogel properties influence ecm production by chondrocytes photoencapsulated in poly (ethylene glycol) hydrogels. *J. Biomed. Mater. Res.* **2002**, *59*, 63-72.
20. Callahan, L.A.S.; Ganios, A.M.; Childers, E.P.; Weiner, S.D.; Becker, M.L. Primary human chondrocyte extracellular matrix formation and phenotype maintenance using RGD-derivatized PEGDM hydrogels possessing a continuous young's modulus gradient. *Acta Biomater.* **2013**, *9*, 6095-6104.
21. Burdick, J.A.; Chung, C.; Jia, X.; Randolph, M.A.; Langer, R. Controlled degradation and

mechanical behavior of photopolymerized hyaluronic acid networks. *Biomacromolecules* **2005**, *6*, 386-391.

22. Schuh, E.; Hofmann, S.; Stok, K.; Notbohm, H.; Müller, R.; Rotter, N. Chondrocyte redifferentiation in 3D: The effect of adhesion site density and substrate elasticity. *J Biomed Mater Res A* **2012**, *100*, 38-47.
23. Jeon, O.; Bouhadir, K.H.; Mansour, J.M.; Alsberg, E. Photocrosslinked alginate hydrogels with tunable biodegradation rates and mechanical properties. *Biomaterials* **2009**, *30*, 2724-2734.
24. Stainsby, G.; WARD, G.; Courts, A. The science and technology of gelatin. *by AG Ward and A. Courts, Academic Press Inc., New York* **1977**, 179.
25. Sell, S.A.; Wolfe, P.S.; Garg, K.; McCool, J.M.; Rodriguez, I.A.; Bowlin, G.L. The use of natural polymers in tissue engineering: A focus on electrospun extracellular matrix analogues. *Polymers* **2010**, *2*, 522-553.
26. Awad, H.A.; Wickham, M.Q.; Leddy, H.A.; Gimble, J.M.; Guilak, F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials* **2004**, *25*, 3211-3222.
27. Lien, S.-M.; Ko, L.-Y.; Huang, T.-J. Effect of pore size on ECM secretion and cell growth in gelatin scaffold for articular cartilage tissue engineering. *Acta Biomater.* **2009**, *5*, 670-679.
28. Schuurman, W.; Levett, P.A.; Pot, M.W.; van Weeren, P.R.; Dhert, W.J.; Hutmacher, D.W.; Melchels, F.P.; Klein, T.J.; Malda, J. Gelatin‐methacrylamide hydrogels as potential biomaterials for fabrication of tissue‐engineered cartilage constructs. *Macromol. Biosci.* **2013**, *13*, 551-561.
29. Luo, Y.; Shoichet, M.S. A photolabile hydrogel for guided three-dimensional cell growth and migration. *Nat. Mater.* **2004**, *3*, 249-253.
30. West, J.L. Protein-patterned hydrogels: Customized cell microenvironments. *Nat. Mater.* **2011**, *10*, 727-729.
31. Benton, J.A.; DeForest, C.A.; Vivekanandan, V.; Anseth, K.S. Photocrosslinking of gelatin macromers to synthesize porous hydrogels that promote valvular interstitial cell function. *Tissue Eng. Part A* **2009**, *15*, 3221-3230.
32. Zhou, D.; Ito, Y. Visible light-curable polymers for biomedical applications. *Sci. China Chem.* **2014**, *57*, 510-521.
33. Wei, D.; Xiao, W.; Sun, J.; Zhong, M.; Guo, L.; Fan, H.; Zhang, X. A biocompatible hydrogel with improved stiffness and hydrophilicity for modular tissue engineering assembly. *J Mater Chem B* **2015**, *3*, 2753-2763.
34. Nichol, J.W.; Koshy, S.T.; Bae, H.; Hwang, C.M.; Yamanlar, S.; Khademhosseini, A. Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials* **2010**, *31*, 5536-5544.
35. Van Den Bulcke, A.I.; Bogdanov, B.; De Rooze, N.; Schacht, E.H.; Cornelissen, M.; Berghmans, H. Structural and rheological properties of methacrylamide modified gelatin hydrogels. *Biomacromolecules* **2000**, *1*, 31-38.
36. Hoch, E.; Hirth, T.; Tovar, G.E.; Borchers, K. Chemical tailoring of gelatin to adjust its chemical and physical properties for functional bioprinting. *J Mater Chem B* **2013**, *1*, 5675-5685.
37. Das, P.; Jana, N.R. Highly colloidally stable hyperbranched polyglycerol grafted red

fluorescent silicon nanoparticle as bioimaging probe. *ACS Appl. Mater. Interfaces* **2014**, *6*, 4301-4309.

38. Smith, R.E.; MacQuarrie, R. A sensitive fluorometric method for the determination of arginine using 9, 10-phenanthrenequinone. *Anal. Biochem.* **1978**, *90*, 246-255.
39. Xiao, W.; He, J.; Nichol, J.W.; Wang, L.; Hutson, C.B.; Wang, B.; Du, Y.; Fan, H.; Khademhosseini, A. Synthesis and characterization of photocrosslinkable gelatin and silk fibroin interpenetrating polymer network hydrogels. *Acta Biomater.* **2011**, *7*, 2384-2393.
40. Zhang, Q.; Lu, H.; Kawazoe, N.; Chen, G. Pore size effect of collagen scaffolds on cartilage regeneration. *Acta Biomater.* **2014**, *10*, 2005-2013.
41. Lu, H.; Ko, Y.-G.; Kawazoe, N.; Chen, G. Cartilage tissue engineering using funnel-like collagen sponges prepared with embossing ice particulate templates. *Biomaterials* **2010**, *31*, 5825-5835.
42. Edep, M.E.; Shirani, J.; Wolf, P.; Brown, D.L. Matrix metalloproteinase expression in nonrheumatic aortic stenosis. *Cardiovasc. Pathol.* **2000**, *9*, 281-286.
43. Kim, I.L.; Khetan, S.; Baker, B.M.; Chen, C.S.; Burdick, J.A. Fibrous hyaluronic acid hydrogels that direct MSC chondrogenesis through mechanical and adhesive cues. *Biomaterials* **2013**, *34*, 5571-5580.
44. Mathieu, P.S.; Loboa, E.G. Cytoskeletal and focal adhesion influences on mesenchymal stem cell shape, mechanical properties, and differentiation down osteogenic, adipogenic, and chondrogenic pathways. *Tissue Eng. Part B: Rev.* **2012**, *18*, 436-444.
45. Moreira Teixeira, L.; Leijten, J.; Sobral, J.; Jin, R.; Apeldoorn, v.A.; Feijen, J.; Blitterswijk, v.C.; Dijkstra, P.; Karperien, H. High throughput generated micro-aggregates of chondrocytes stimulate cartilage formation in vitro and in vivo. *Eur. cells Mater.* **2012**, *23*, 387-399.
46. Fang, J.; Yong, Q.; Zhang, K.; Sun, W.; Yan, S.; Cui, L.; Yin, J. Novel injectable porous poly (γ-benzyl-l-glutamate) microspheres for cartilage tissue engineering: Preparation and evaluation. *J Mater Chem B* **2015**, *3*, 1020-1031.
47. Liu, Y.; Chan-Park, M.B. A biomimetic hydrogel based on methacrylated dextran-graft-lysine and gelatin for 3D smooth muscle cell culture. *Biomaterials* **2010**, *31*, 1158-1170.
48. Lv, H.; Li, L.; Sun, M.; Zhang, Y.; Chen, L.; Rong, Y.; Li, Y. Mechanism of regulation of stem cell differentiation by matrix stiffness. *Stem Cell Res. Ther.* **2015**, *6*, 103.
49. Allen, J.L.; Cooke, M.E.; Alliston, T. ECM stiffness primes the TGFβ pathway to promote chondrocyte differentiation. *Mol. Biol. Cell* **2012**, *23*, 3731-3742.
50. Chung, C.; Burdick, J.A. Influence of three-dimensional hyaluronic acid microenvironments on mesenchymal stem cell chondrogenesis. *Tissue Eng. Part A* **2008**, *15*, 243-254.
51. Acharya, C.; Adesida, A.; Zajac, P.; Mumme, M.; Riesle, J.; Martin, I.; Barbero, A. Enhanced chondrocyte proliferation and mesenchymal stromal cells chondrogenesis in coculture pellets mediate improved cartilage formation. *J. Cell. Physiol.* **2012**, *227*, 88-97.
52. Marklein, R.A.; Burdick, J.A. Controlling stem cell fate with material design. *Adv. Mater.* **2010**, *22*, 175-189.
53. Chen, S.; Zhang, Q.; Nakamoto, T.; Kawazoe, N.; Chen, G. Gelatin scaffolds with controlled pore structure and mechanical property for cartilage tissue engineering. *Tissue Eng. Part C: Methods* **2016**.

54. Zhang, K.; Wang, L.; Han, Q.; Heng, B.C.; Yang, Z.; Ge, Z. Relationship between cell function and initial cell seeding density of primary porcine chondrocytes in vitro. *Biomed. Eng. Appl. Basis Commun.* **2013**, *25*, 1340001.
55. Khetan, S.; Guvendiren, M.; Legant, W.R.; Cohen, D.M.; Chen, C.S.; Burdick, J.A. Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels. *Nat. Mater.* **2013**, *12*, 458-465.
56. Chaudhuri, O.; Gu, L.; Klumpers, D.; Darnell, M.; Bencherif, S.A.; Weaver, J.C.; Huebsch, N.; Lee, H.-p.; Lippens, E.; Duda, G.N., *et al.* Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat Mater* **2016**, *15*, 326-334.
57. Gao, Y.; Liu, S.; Huang, J.; Guo, W.; Chen, J.; Zhang, L.; Zhao, B.; Peng, J.; Wang, A.; Wang, Y. The ECM-cell interaction of cartilage extracellular matrix on chondrocytes. *Biomed. Res. Int.* **2014**, *2014*.
58. Zhang, Z. Chondrons and the pericellular matrix of chondrocytes. *Tissue Eng. Part B Rev.* **2014**, *21*, 267-277.
59. Alexopoulos, L.G.; Setton, L.A.; Guilak, F. The biomechanical role of the chondrocyte pericellular matrix in articular cartilage. *Acta Biomater.* **2005**, *1*, 317-325.
60. Buxboim, A.; Ivanovska, I.L.; Discher, D.E. Matrix elasticity, cytoskeletal forces and physics of the nucleus: How deeply do cells ‘feel’outside and in? *J Cell Sci* **2010**, *123*, 297-308.
61. Koh, W.-G.; Revzin, A.; Pishko, M.V. Poly (ethylene glycol) hydrogel microstructures encapsulating living cells. *Langmuir* **2002**, *18*, 2459-2462.
62. Anderson, S.B.; Lin, C.-C.; Kuntzler, D.V.; Anseth, K.S. The performance of human mesenchymal stem cells encapsulated in cell-degradable polymer-peptide hydrogels. *Biomaterials* **2011**, *32*, 3564-3574.

Chapter 3

Fabrication of highly crosslinked gelatin hydrogel and its influence on chondrocyte proliferation and phenotype

3.1 Summary

Gelatin methacrylate (GelMA) hydrogels have been widely studied for biomedical applications, such as tissue engineering and drug delivery, because of their good biocompatibility and injectability. However, the quick degradation and low mechanical property of GelMA hydrogels need to be improved for further applications, especially for long-term implantation. In this part, a sequential double modification of gelatin was used to achieve high density of photocrosslinkable double bonds in gelatin derivatives. The amino groups in gelatin were first reacted with methacrylic anhydride. After this, the hydroxyl and carboxyl groups in gelatin were reacted with glycidyl methacrylate to obtain the double modified gelatin macromer. The double modified gelatin macromer was used to prepare gelatin hydrogels with high crosslinking density. The hydrogels exhibited high storage modulus and low degradation. Culture of bovine articular chondrocytes in the gelatin hydrogels showed that chondrocytes had round morphology and maintained a cartilaginous phenotype while cell proliferation was hampered. This method for increasing crosslinking density should be useful for preparation of stable hydrogels for cartilage tissue engineering.

3.2 Introduction

Cartilage tissue engineering has drawn a lot of attention due to the limited self-repairing capacity of avascular cartilage. One crucial step of cartilage tissue engineering is *in vitro* expansion of chondrocytes, during which chondrocytes lose part of their chondrogenic phenotype due to the differences between the two-dimensional (2D) expansion culture microenvironment and the *in vivo* three-dimensional (3D) microenvironment. The loss of expression of cartilaginous matrices, such as collagen type II and aggrecan is a process known as de-differentiation. De-differentiated chondrocytes should be re-differentiated in order to express cartilaginous matrices for functional cartilage tissue engineering.[1] Hydrogels have been frequently

used for the 3D culture of chondrocytes for re-differentiation and maintenance of chondrogenic phenotype and for chondrogenic differentiation of stem cells.[2-4] Hydrogels are hydrated polymers with 3D network structures that are similar to the microenvironments surrounding chondrocytes *in vivo*.[5] Many reports have shown the re-differentiation of de-differentiated chondrocytes in hydrogels.[6] Among the hydrogels, gelatin hydrogels have many advantages, such as good cell adhesion (RGD peptide), good biocompatibility and easy modification.[7-11]

Gelatin is usually modified with methacrylate groups for gelatin hydrogel preparation. The main limitations of GelMA hydrogel for tissue engineering, especially for the tissues requiring extensive load-bearing properties, are its poor mechanical properties and short degradation time.[12,13] The mechanical properties and degradation of hydrogels are related to hydrogel network crosslinking density.[14] The compressive modulus increases as the crosslinking density increases.[15] Altering precursor macromer concentration has been reported to modulate crosslinking density of hydrogels.[16] However, altering macromer concentration cannot decouple the influence of hydrogel matrix concentration. It is particularly important for gelatin hydrogel because gelatin holds RGD sequences and different gelatin concentrations may result in different densities of RGD. Other methods to increase crosslinking density include prolonging of crosslinking time.[17] However, long UV irradiation time may decrease cell viability. Incorporation of more photo-reactive double bonds to increase the ratio of photocrosslinkable bonds to photo-initiator during hydrogel preparation has been proposed as an attractive method to increase crosslinking density because a high ratio of photocrosslinkable bonds to photo-initiator can protect cells from free radical attack and therefore lead to high cell viability.[18]

For the methacrylation of gelatin molecules, amino groups are usually used for reaction with methacrylate groups. However, the number of amino groups in each gelatin molecule is limited. There are many hydroxyl and carboxyl groups in gelatin molecules. Therefore, usage of these functional groups for further methacrylation of gelatin is desirable to increase the crosslinking density of gelatin hydrogels.

In this study, a sequential double modification of amino, hydroxyl and carboxyl groups of gelatin molecules was used to introduce more photocrosslinkable methacrylate groups in gelatin for the preparation of gelatin hydrogels with a high crosslinking density. The elastic property and enzymatic degradation rate of the hydrogel were evaluated. The gelatin hydrogels were used for 3D culturing of chondrocytes, and their effects on cell proliferation and expression of cartilaginous matrices were investigated.

3.3 Materials and methods

3.3.1 Synthesis of GelMA and GelMAGMA

GelMA macromer was synthesized according to a previously described method.[7] Briefly, 5 g gelatin (type A, 300 bloom, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate buffered saline (PBS) at 50 ^{o}C under stirring to obtain a 10% (w/v) gelatin solution. 5 mL methacrylic anhydride (MA; Sigma-Aldrich, St. Louis, MO, USA) was added into the gelatin solution at a rate of 0.5 mL/minute while stirring at 50 ^{o}C to prepare GelMA. After reaction in the dark for 3 hours, the product was diluted with five-fold warm PBS (50 ^{o}C) and then dialyzed against Milli-Q water for seven days at 40 ^{o}C using a dialysis membrane (12-14 kD molecular weight cut-off, Spectrum Laboratories Inc. USA) to remove salts and excess free MA. The GelMA foam was obtained after freeze-drying for two days.

Glycidyl methacrylate-modified GelMA (GelMAGMA) macromer was synthesized by modifying the

GelMA macromer with glycidyl methacrylate.[19] After dissolving 2.5 g of GelMA macromer in Milli-Q water (2%, w/v), the pH of the solution was adjusted to 3.5 with 1 M HCl (Wako, Osaka, Japan). After this, 5 mL of glycidyl methacrylate (GMA; Sigma-Aldrich, St. Louis, MO, USA) was added into the solution at a rate of 0.5 mL/minute.[20] The reaction was conducted at 50 ºC for 24 hours and the product was purified by dialysis against Milli-Q water with the above-mentioned dialysis membrane at 40 ºC for seven days. The purified product was freeze-dried and stored at -20 ºC for further use.

3.3.2 ^{1}H nuclear magnetic resonance (NMR)

The degree of functionalization was studied by using ^{1}H NMR according to a previously reported method.[21] ^{1}H NMR spectra were collected by using an NMR spectrometer (AL300; JEOL, Tokyo, Japan) with a single axis gradient inverse probe at a frequency of 300 MHz. Before the measurement, 30 mg of GelMA and GelMAGMA macromers were respectively dissolved into 1 mL deuterium oxide containing 0.05% (w/v) 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (Sigma-Aldrich, St. Louis, MO, USA) for calibration at 40 ºC.

3.3.3 Preparation of GelMA and GelMAGMA hydrogels

GelMA and GelMAGMA solutions were prepared by dissolving the respective macromers (10%, w/v) and photoinitiator, 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (I 2959, Sigma-Aldrich, St. Louis, MO, USA), (0.5%, w/v) in PBS solution at 50 ºC. The above solutions were sterilized by filtration through a 0.22 µm filter (Millex-GV, Carrigtohill, Cork, Ireland). After sterilization, the solution was pipetted between two sterile quartz glass coverslips separated by a sterile silicone membrane frame with a 1.5 mm thickness (KOKEN Co., Ltd., Tokyo, Japan) and exposed to UV light (CL-1000, Funakoshi Co., Ltd., Japan) for 5 minutes. After cross-linking, the hydrogels were punched into disks with a 10 mm biopsy punch (Acu-Punch, Acuderm Inc, Fla, USA) for rheological testing and a 6 mm biopsy punch (Kai Medical, Gifu, Japan) for the other experiments.

3.3.4 Rheological measurements

The storage modulus of GelMA and GelMAGMA hydrogel disks (10 mm of diameter) were measured by an MCR301 Rheometer (Anton Paar, Germany) equipped with 10 mm parallel plates. The temperature was set at 37 ºC, and the samples were balanced for 3 minutes before the start of testing. The storage moduli of GelMA and GelMAGMA hydrogels were measured by an oscillatory shear deformation at a constant frequency (1 Hz) and a constant shear strain (5%).[21] Three samples were tested to calculate means and standard deviations.

3.3.5 Swelling ratio and enzymatic degradation of the hydrogels

GelMA and GelMAGMA hydrogel disks (6 mm of diameter) were swollen in PBS for 24 hours to reach swelling equilibrium. The samples were blotted with a KimWipe paper to remove the residual liquid and weighed to obtain the equilibrium wet weight. The dry weight was measured after freeze-drying. The swelling ratio was calculated according to a previous report.[22] Three samples were used for the

measurement to calculate means and standard deviations.

The swollen hydrogel disks were immersed in 5 ml of PBS containing 10 units mL^{-1} of collagenase I (Worthington Biochemical, Lakewood, NJ, USA) and incubated at 37 ºC in an orbital shaker at a shaking speed of 100 rpm. At the designated time points, the hydrogel disks were taken out and weighed after the removal of the excess solution by blotting. The degradation degree of the hydrogels was calculated by normalizing the residual hydrogel wet weight to the initial wet weight. Five samples were used at every time point for the measurement to calculate means and standard deviations.

3.3.6 Chondrocytes isolation and culture in vitro

Bovine articular chondrocytes (BACs) were isolated from articular cartilage from the knees of a calf according to previously reported protocol.[23] Briefly, the articular cartilage tissue was minced into small pieces using a sterile surgery scalpel. The minced tissue pieces were digested with a 0.2% w/v collagenase type II (Worthington Biochemical, Lakewood, NJ, USA) aqueous solution overnight at 37 ºC with shaking. The digestion solution was filtered through a sterile nylon mesh with a 70 µm mesh size to remove any undigested fragments. The isolated primary chondrocytes were collected by centrifugation and cultured in 75 cm^2 tissue culture flasks in Dulbecco's Modified Eagle Medium (D6546; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 4500 mg L^{-1} glucose, 4 mM glutamine, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 1 mM sodium pyruvate and 50 µg mL^{-1} ascorbic acid at 37 ºC and 5% CO_2. The culture medium was refreshed every three days. The cells were subcultured after reaching confluence. The chondrocytes at passage 2 were used in the following experiments. The cells were detached with a trypsin/EDTA solution, collected by centrifugation, counted with a hemocytometer and re-suspended in the mixture solution of GelMA or GelMAGMA macromer (10%, w/v) and photo-initiator (0.5 %, w/v) sterilized by 0.22 µm filter. The cell suspension solution was added between two quartz glass coverslips separated by a 1.5 mm-thick silicone membrane frame and exposed to UV light to prepare cell-laden GelMA or GelMAGMA hydrogel as described in section 2.3. The quartz glass and silicone frame were sterilized by 70% ethanol and washed with PBS. The cell-laden hydrogels were punched into disks with a 6 mm biopsy punch. Before culturing, all the samples were washed twice to remove the photo-initiator by immersing in culture medium for 30 minutes. The cell-laden hydrogel disks were transferred to cell culture T-flasks for dynamic cell culture under shaking (60 rpm). The culture medium was changed every two days. All the procedures were conducted under sterile conditions on a clean bench. The UV crosslinking machine was sterilized by 70% ethanol and moved into the clean bench before UV crosslinking. As a two-dimensional culture control, the passage 2 chondrocytes were seeded in 24-well tissue culture plates (TCP) and cultured with the same medium as that used for three-dimensional culture in the hydrogels.

3.3.7 Cell viability assay

Live/dead staining was performed to evaluate cell viability of chondrocytes in the hydrogels by using Cellstain Double Staining Kit (Dojindo Laboratories, Tokyo, Japan). After UV crosslinking and 14 days of *in vitro* culture, the cell-laden hydrogel disks were washed with PBS for three times and incubated with serum-free medium containing calcein-AM (2 µM) and propidium iodide (4 µM) at 37 ºC for 15 minutes. The stained cells were observed using a confocal microscope (Zeiss LSM 510 Meta).

3.3.8 In vivo implantation

All the animal experiment procedures were approved by the Animal Experiments Committee of the National Institute for Materials Science and the experiment was conducted according to the committee guidelines. After the cell-laden hydrogel disks were cultured in the culture medium under shaking for one day, they were subcutaneously implanted in the dorsa of athymic nude mice. After six weeks of implantation, the mice were euthanized and the implants were harvested for further study.

3.3.9 Quantification of DNA and sulfated glycosaminoglycan (sGAG)

DNA amount and sGAG content in the hydrogel disks were quantified after six weeks of implantation. The harvested cell-laden hydrogel disk implants were washed with PBS three times and freeze-dried. Each of the freeze-dried implants was digested by 500 μL papain solution (Sigma-Aldrich, St. Louis, MO, USA), which was prepared by dissolving papain at a concentration of 400 mg/mL in 0.1 M PBS (pH 6.0) containing 5 mM cysteine hydrochloride and 5 mM ethylenediaminetetraacetic acid (EDTA). 5 μL of the papain digestion solution was used to measure the DNA amount with Hoechst 33258 dye (Sigma-Aldrich, St. Louis, MO, USA). The fluorescence intensity was read with an FP-6500 spectrofluorometer (JASCO, Tokyo, Japan) at an excitation/emission wavelength of 360 nm and 460 nm. The sGAG content in each digestion solution was measured by using a BlyscanTM Glycosaminoglycan Assay Kit (Biocolor Ltd., County Antrim, UK). Four samples in each group were used for the measurement to calculate means and standard deviations.

3.3.10 Histological and immunohistochemical staining

The harvested cell-laden hydrogel disk implants were washed three times with PBS and fixed with 10% neutral buffer formalin (Wako, Osaka, Japan) at room temperature for two days. After that, the implants were dehydrated in a series of ethanol solutions with increasing ethanol concentration from 70% to 99.5%, embedded in paraffin and sectioned with a microtome (Leica RM2245; Germany) to obtain cross-sections having a 7 μm thickness. The cross-sections were then de-paraffinized and stained with hematoxylin and eosin (HE) for cell morphology; and safranin O/fast green and alcian blue for glycosaminoglycan. Briefly, the de-paraffinized sections were immersed in hematoxylin solution for 10 minutes, and then washed with tap water for 10 min before immersing in eosin solution for 3 minutes. For safranin O/fast green staining, the de-paraffinized sections were stained with 0.02% aqueous solution of fast green for 1 minute, followed by washing with tap water, immersion in 1% acetic acid aqueous solution for 15 seconds and immersion in 0.1% safranin O aqueous solution for 10 minutes. The alcian blue staining was conducted by immersing the de-paraffinized sections in alcian blue aqueous solution for 30 minutes. The immunohistochemical staining of collagen type II and collagen type I was performed according to a previous report.[24] Briefly, the de-paraffinized sections were incubated with proteinase K (Dako Corp., Carpinteria, CA, USA) for 10 minutes for antigen retrieval, and then incubated with peroxidase blocking solution (Dako) for 5 minutes and 10% goat serum solution (Dako) for 30 minutes. Next, the sections were incubated with the first antibodies for 2 hours at room temperature, followed by incubation with the peroxidase-labeled polymer-conjugated second antibodies (DakoCytomation Envision+, Dako, Carpinteria, CA, USA) for another 30 minutes at room temperature. The first antibodies were rabbit polyclonal anti-collagen type II at a 1:100 working dilution (AB746; Millipore, Schwalbach, Germany) and rabbit monoclonal anti-collagen type I at a 1:100

working dilution (AB138492; Abcam, Cambridge, MA, USA). The second antibody was HRP-labeled polymer conjugated secondary antibody (anti-rabbit; Dako, Carpinteria, CA, USA). The sections were finally incubated with 3,3'-diaminobenzidine (DAB; Dako) for 10 minutes to develop color. The chondrocytes cultured in 24-well tissue culture plates were stained with the same protocols after reaching confluence by five days of culturing. The stained samples were observed under an optical microscope.

3.3.11 Real-time PCR analysis

Expression of genes encoding collagen type II, aggrecan, Sox 9 and collagen type I were analyzed by a real-time PCR.[25] The cell-laden hydrogel disk implants were washed with PBS three times, frozen in liquid nitrogen and crushed into powder by an electric crusher. The powder samples were dissolved in Sepasol solution (1 mL per sample; Nacalai tesque, Kyoto, Japan) to isolate the RNA. The RNA content in each sample was quantified, after which the RNA was converted to cDNA by MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). Real-time PCR was used to amplify glyceraldehyde-3-phosphate dehydrogenase (Gapdh), type II collagen (Col-II), aggrecan (Acan), Sox 9 and type I collagen (Col-I) by using a 7500 real-time PCR System (Applied Belgium, Foster City, CA, USA). The expression level of Gapdh, a housekeeping gene, was used as an endogenous control. The relative expression of the target gene was calculated by using the $2^{-\Delta\Delta C_T}$ formula and passage 2 chondrocytes as a reference. The primer and probe sequences were the same as those used in the previous study. Four samples in each group were used for the measurement to calculate means and standard deviations.

3.3.12 Statistical analysis

All data were reported as the mean ± standard deviation (SD). Statistical analysis was performed using a one-way ANOVA analysis to evaluate the significance of the experimental data. Statistical differences were considered significant if $p < 0.05$. (*), (**) and (***) indicated $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. All statistical analyses were executed by using KyPlot 2.0 beta 15 (1997–2001 Koichi Yoshioka).

3.4 Results and discussion

3.4.1 Synthesis and ^{1}H NMR of GelMA and GelMAGMA macromers

Gelatin has abundant amino, hydroxyl and carboxyl groups that can be used for modification. After the reaction of the amino groups in gelatin molecules with methacrylic anhydride, the remaining hydroxyl and carboxyl groups can be used for further modification in an acidic environment.[20] GelMA macromer was first synthesized, and then the GelMA macromer was used for the second modification to synthesize GelMAGMA. The ^{1}H NMR spectra of GelMA and GelMAGMA macromers are shown in Figure 3.1a. The protons in methacrylate groups are indicated in the ^{1}H NMR spectra. Integrated intensity of the protons in methacrylate groups of the GelMAGMA macromer (1.83) was much higher than that of the GelMA macromer (0.96). The result indicated that the methacrylate group density in the GelMAGMA macromer was higher than that in the GelMA macromer. The second modification by reaction between the hydroxyl and carboxyl groups with glycidyl methacrylate increased the photocrosslinkable double bonds in the GelMAGMA

macromer, which should result in high degree of cross-linking in the GelMAGMA hydrogels. The GelMA and GelMAGMA macromers were used to prepare gelatin hydrogels having respectively low and high densities of cross-linking. Chondrocytes were suspended in the macromer solution before UV irradiation to prepare cell-laden gelatin hydrogels (Figure 3.1b).

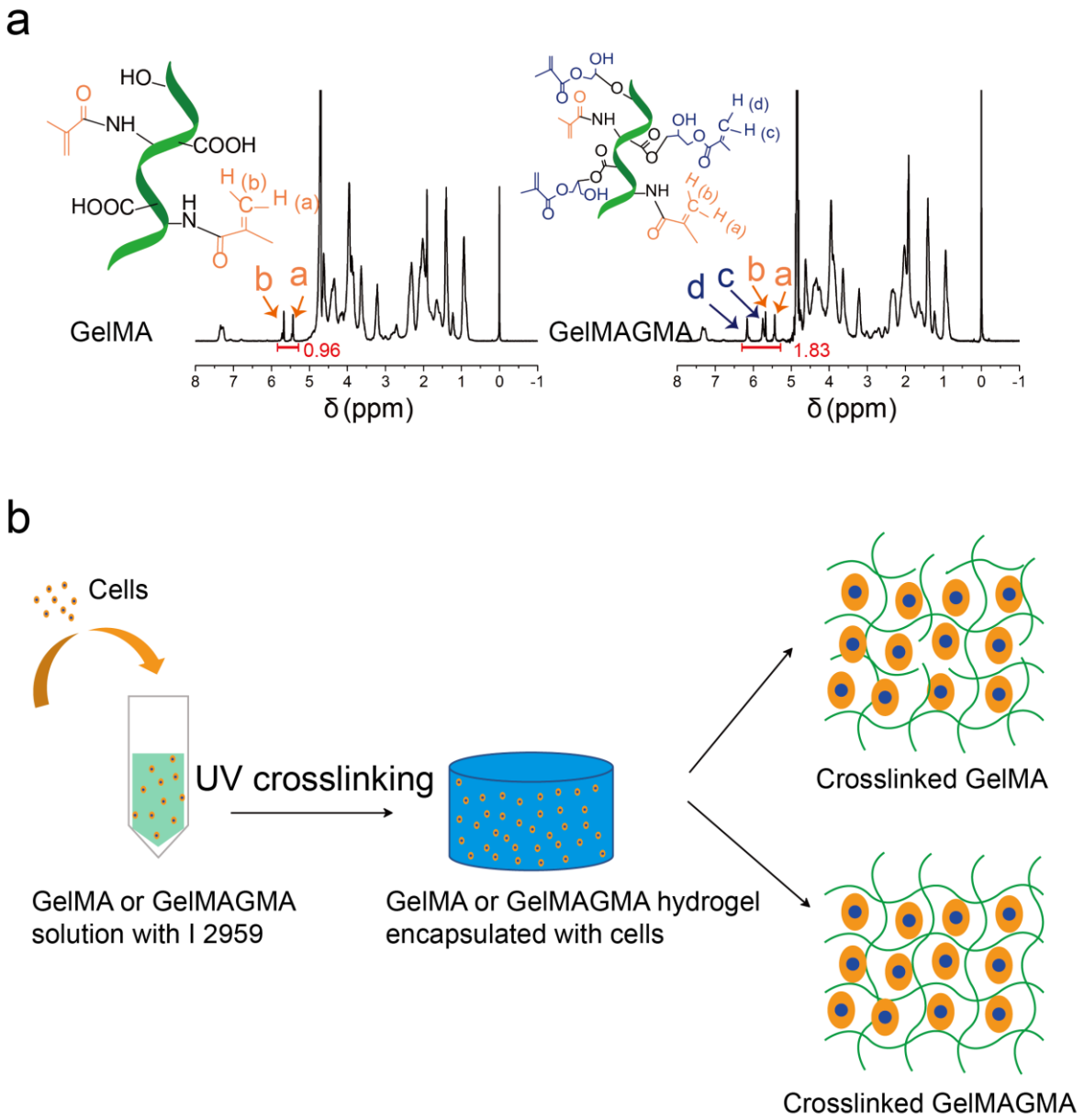

Figure 3.1 ^{1}H NMR spectra of GelMA and GelMAGMA macromers (a). Experimental scheme of GelMA and GelMAGMA hydrogels encapsulated with chondrocytes and illustration of cell-laden hydrogel networks (b).

3.4.2 Rheological property of GelMA and GelMAGMA hydrogels

The GelMA solution became hydrogel at room temperature, while the GelMAGMA solution kept its solution state even at room temperature (Figure 3.2a), indicating a reduction of the intermolecular interaction forces of the gelatin chains by modification.[21]

The storage modulus (G') of the GelMA and GelMAGMA hydrogels (10%, w/v) was measured at a constant deformation of 5% and a frequency of 1 Hz (Figure 3.2b). The storage modulus of the GelMAGMA hydrogel was significantly higher than that of the GelMA hydrogel. This should be due to high crosslinking density and steric hindrance between polymer chains in the GelMAGMA hydrogel. It has been reported that compressive modulus of a GelMA hydrogel increases with the degree of modification.[26] Hydrogels with different modification degrees and concentrations of GelMA have been reported to exhibit a broad range of compressive moduli from 3 to 30 kPa.[7] Hydrogels with 30 kPa Young's modulus can be formed with 15% GelMA having a modification degree of 80%. In our previous study, the compressive modulus of GelMA hydrogel could be enhanced to 30 kPa by using 10% GelMA macromer having a modification degree of 90%.[9] The GelMAGMA hydrogel in this study showed a higher storage modulus than did the GelMA hydrogel. The results indicated that the stiffness of gelatin-based hydrogel could be increased by sequential modification with methacrylic anhydride and glycidyl methacrylate without changing gelatin concentration. It has been reported that the different properties of crosslinked hydrogels, such as storage modulus, swelling ratio and degradation rate can be controlled by network crosslinking density.[14] It has also been reported that the mechanical property of GelMA hydrogel can be adjusted by modification degree or macromer concentration.[7,9] The sequential double modification of amino, hydroxyl and carboxyl groups in gelatin molecules was demonstrated as an efficient way to adjust the crosslinking density and Young's modulus of gelatin hydrogels.

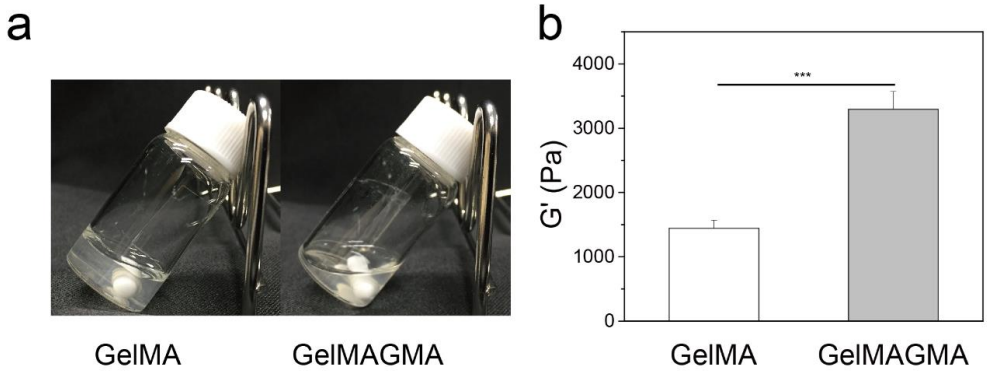

Figure 3.2 Gross appearance of GelMA and GelMAGMA solution (10%, w/v) at room temperature (a). The storage modulus (G') of GelMA and GelMAGMA hydrogels at 37 ºC with a constant deformation of 5% and a frequency of 1 Hz (b). The data represent means ± SD, N=3.

3.4.3 Swelling behavior and enzymatic degradation

The swelling ratio of hydrogels is controlled by polymer network mesh size and polymer-solvent interaction. The swelling ratio of GelMAGMA hydrogel was slightly lower than that of GelMA hydrogel, although the difference was not significant (Figure 3.3a). The low swelling ratio of GelMAGMA hydrogel is most likely due to its high crosslinking density.

Enzymatic degradation showed that the GelMAGMA hydrogel was more stable than the GelMA hydrogel (Figure 3.3b). The GelMA hydrogel was degraded rapidly by collagenase I. In contrast, the GelMAGMA hydrogels showed a very slow degradation rate. Gelatin hydrogels could be degraded by collagenase I easily while crosslinking inhibited its degradation. A higher crosslinking density led to a slower degradation rate. The slow degradation rate of GelMAGMA hydrogel should allow for long-term implantation.

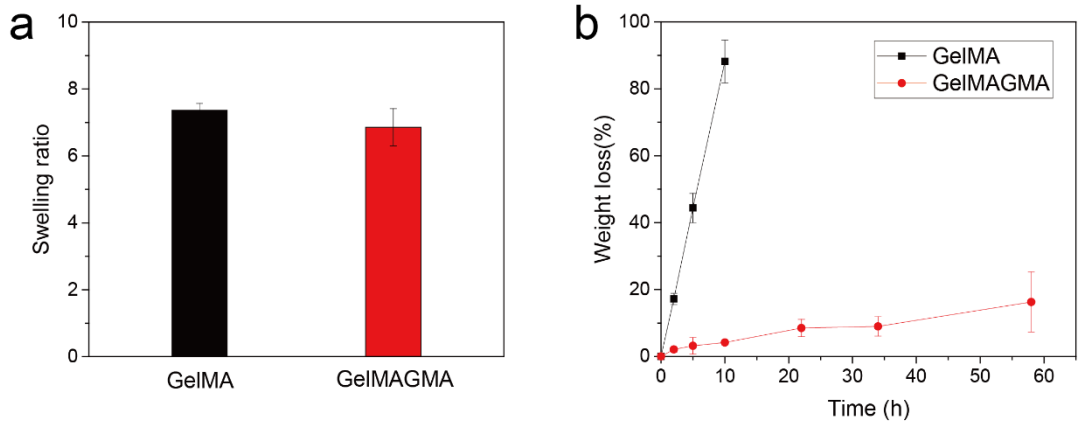

Figure 3.3 Equilibrium swelling ratio of GelMA and GelMAGMA hydrogels (a), the data represent means ± SD, N=3. Enzymatic degradation of GelMA and GelMAGMA hydrogels in the presence of 10 unit mL^{-1} of collagenase type I at 37 ^{o}C with shaking (b), the data represent means ± SD, N=5.

3.4.4 Cell viability in the hydrogels during in vitro culture

Live/dead staining was used to evaluate cell viability in the two kinds of hydrogels immediately after UV crosslinking (Day 0) and after two weeks of *in vitro* culturing (Day 14) (Figure 3.4). A small number of chondrocytes were detected dead after UV-initiated polymerization. After culturing for two weeks, chondrocytes in all the groups showed high cell viability. Some chondrocytes integrated to form small aggregates. Chondrocytes in the GelMA and GelMAGMA hydrogels showed similar results. The results indicated that the UV-initiated polymerization of photocrosslinkable macromers did not evidently affect cell viability and that the GelMA and GelMAGMA hydrogels were compatible for chondrocyte culturing.

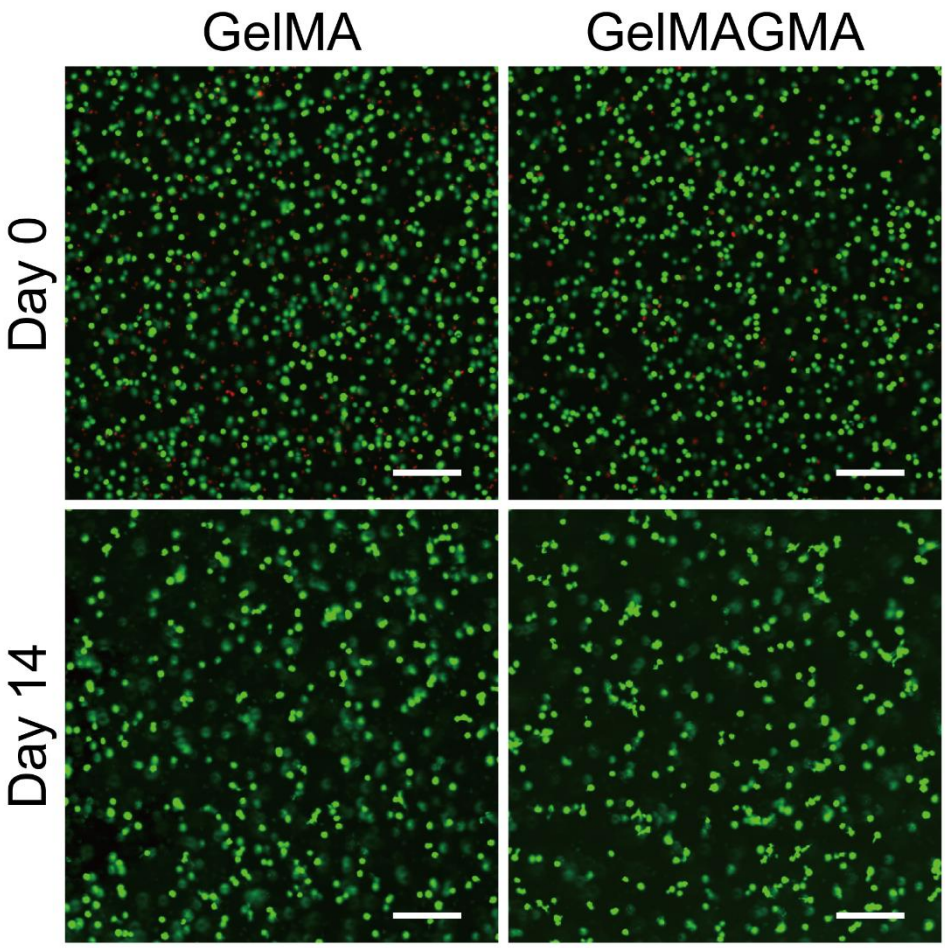

Figure 3.4 Live and dead staining of chondrocytes in GelMA and GelMAGMA hydrogels after UV crosslinking (Day 0) and after 14 days *in vitro* culturing (Day 14). Scale bar = 200 μm. (Green: live cells; Red: dead cells)

3.4.5 DNA and sGAG quantification

DNA amount and sGAG content in the cell-laden hydrogel disk implants at the beginning (week 0) and after six weeks implantation (week 6) were measured (Figure 3.5). After six weeks of implantation, the DNA amount decreased in both GelMA and GelMAGMA hydrogels. However, the sGAG content increased significantly after six weeks of implantation. sGAG is one of the main components of native cartilage and is used to evaluate chondrogenic activity.[27] The decrease of DNA amount might be due to partial cell death and limited nutrient diffusion and penetration in the hydrogels. Formation of cartilaginous matrices in hydrogel has been reported to hamper nutrient diffusion and therefore affect cell proliferation during long period implantation.[28] The high sGAG content indicated the hydrogels were beneficial for secretion of cartilaginous matrices, in particular, the GelMA hydrogel.

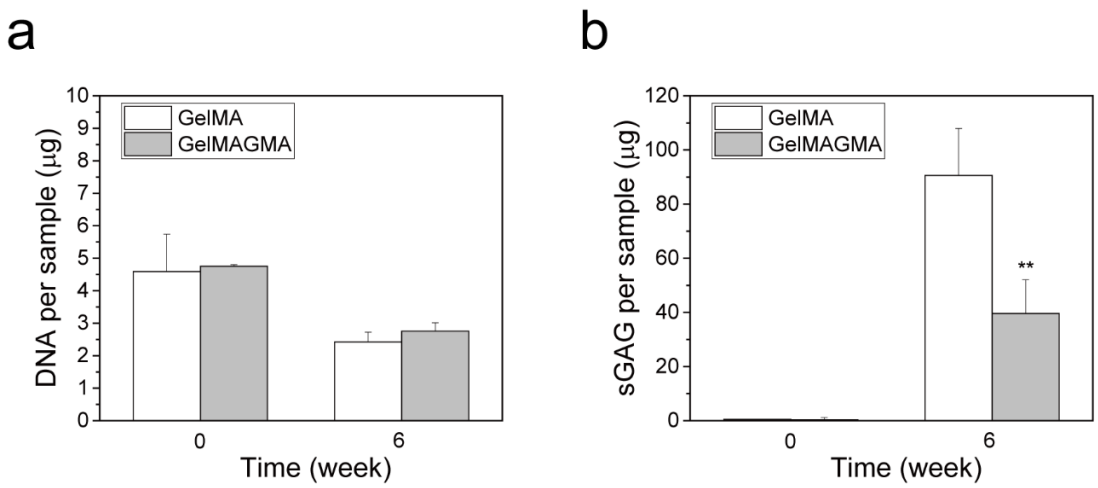

Figure 3.5 DNA amount (a) and sGAG content (b) in GelMA and GelMAGMA hydrogels before implantation (0 week) and after *in vivo* implantation for six weeks (6 week). Means ± SD, N=4.

3.4.6 Histological and immunohistochemical stainings

HE staining showed that chondrocytes in both GelMA and GelMAGMA hydrogels had round morphology that were similar to chondrocyte morphology in native cartilage (Figure 3.6). It has been reported that round cell morphology, possessing weak actin cytoskeletal organization, is beneficial for chondrogenesis.[29] Partial chondrocytes aggregated to form small aggregates. Cell aggregation has been reported to be good for chondrogenic ECM secretion and chondrocyte phenotype maintenance.[30,31] Safranin O and alcian blue staining results showed that cartilaginous matrices were detected around the round cells. Immunohistochemical staining of type II collagen also demonstrated the presence of type II collagen in areas surrounding the round cells. Both GelMA and GelMAGMA hydrogels showed slight staining of type I collagen. The chondrocytes in GelMA hydrogels showed slightly stronger staining of chondrogenic matrices than did the cells in GelMAGMA hydrogels. The high crosslinking density in GelMAGMA hydrogels might inhibit the diffusion of cartilaginous matrices. It has been reported that diffusion of ECM is related to the hydrogel network structure and diffusivity decreases when the crosslinking density increases.[32]

To further compare the morphology and phenotype of chondrocytes in 2D and 3D, the same chondrocytes (passage 2) were cultured in cell culture plates for five days. The chondrocytes reached confluence after five days of culturing. The chondrocytes showed elongated morphology. Safranin O and alcian blue staining showed that no cartilaginous matrices were detected. Immunohistochemical staining of type I and type II collagen showed that type I collagen was detected while no type II collagen was detected. Many studies have reported that chondrocytes change their morphology from round to spindle-like elongated shape and lose the capacity to express cartilaginous matrices during 2D expansion culturing.[33-35]

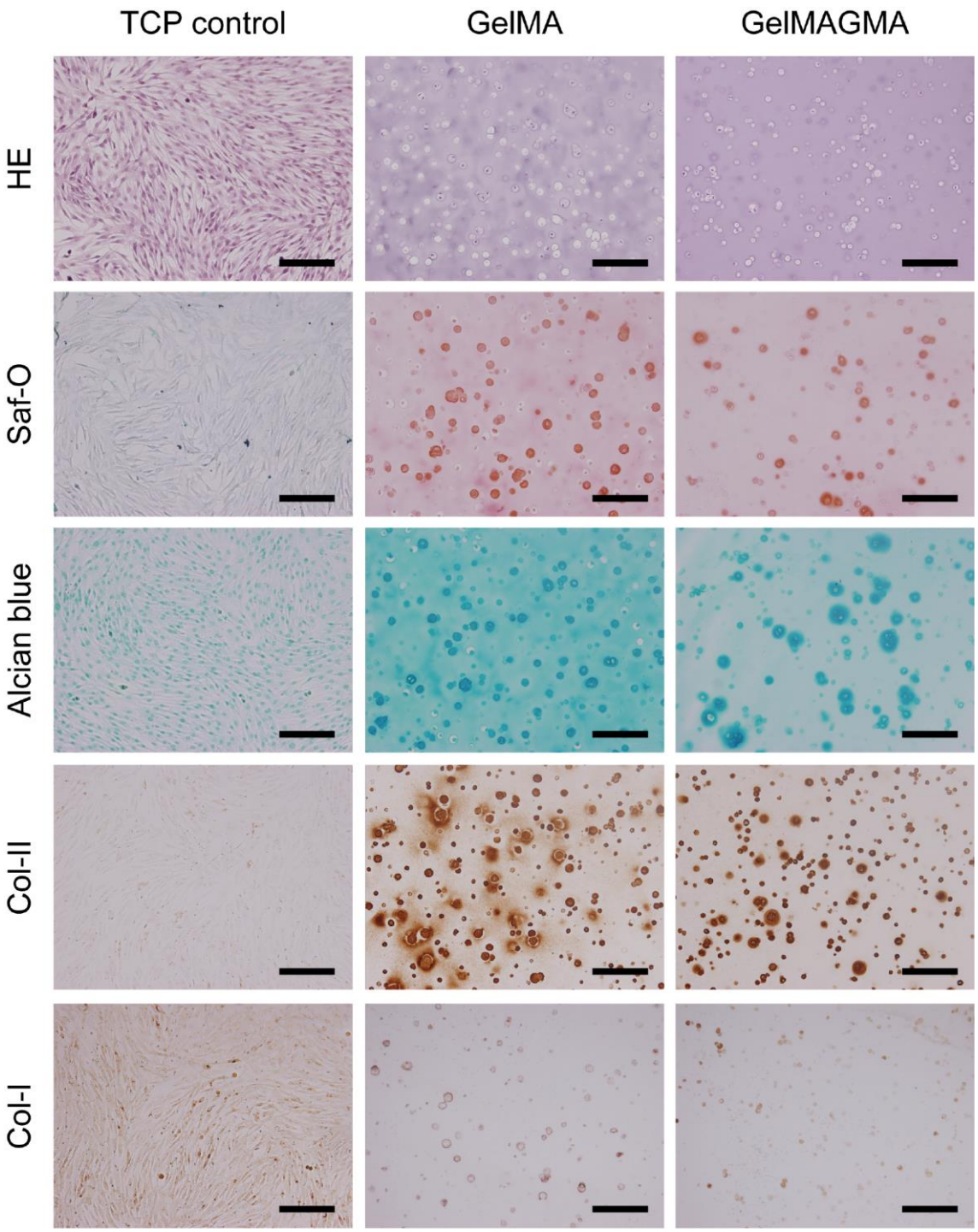

Figure 3.6 Hematoxylin and eosin (HE), safranin O (Saf-O) and alcian blue staining and immunohistochemical staining of collagen type II (Col-II) and collagen type I (Col-I) of the GelMA and GelMAGMA hydrogel implants after subcutaneous implantation for six weeks. Chondrocytes cultured in 24-well tissue culture plates (TCP) for five days were stained as a control. Scale bar = 200 μm.

3.4.7 Cartilaginous gene expression

To further compare the influence of GelMA and GelMAGMA hydrogels on chondrocyte phenotype, gene expression of aggrecan (Figure 3.7a), collagen type II (Figure 3.7b), Sox9 (Figure 3.7c) and collagen

type I (Figure 3.7d) were investigated by real-time PCR. Collagen type II and aggrecan are the two main characteristic genes related to chondrogenic differentiation.[36] Sox9 is the key transcription factor of chondrogenesis and chondrogenic differentiation.[37] The expression level of collagen type II, aggrecan and Sox9 was significantly higher than that of chondrocytes used for cell seeding, while there was no significant difference in expression of collagen type I. The results indicated that culturing in the hydrogels up-regulated cartilaginous gene expression. The hydrogels could provide a 3D microenvironment for expression of cartilaginous genes. The expression level of the cartilaginous genes in the GelMA hydrogel was significantly higher than that in the GelMAGMA hydrogel.

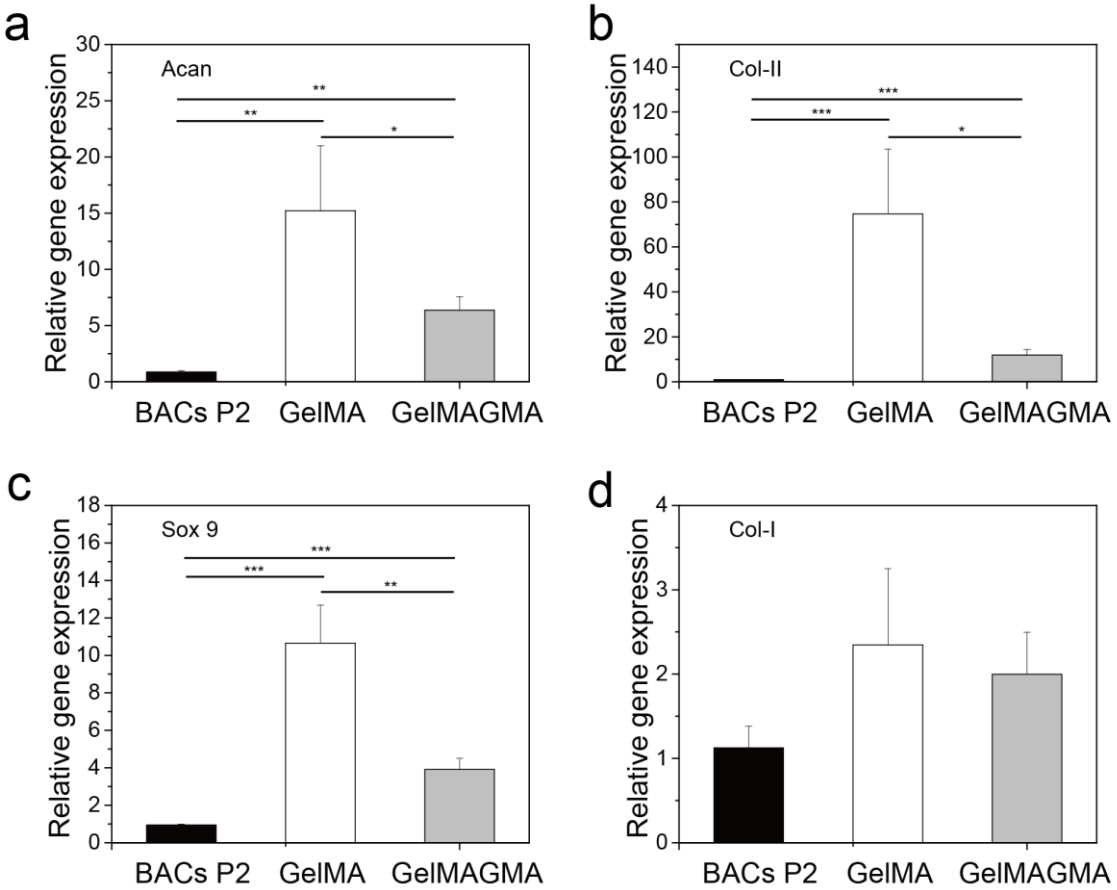

Figure 3.7 Expression of genes encoding aggrecan (a), collagen II (b), Sox 9 (c) and collagen I (d) of chondrocytes in GelMA and GelMAGMA hydrogel implants after six weeks of implantation. Data represent means ± SD, N=3.

In this study, two types of gelatin hydrogels were prepared by modifying the amino, hydroxyl and carboxyl groups in gelatin molecules with photocrosslinkable methacrylate groups. The first modification was conducted by reacting amino groups in gelatin molecules with methacrylic anhydride. The second modification was carried out by coupling the hydroxyl and carboxyl groups with glycidyl methacrylate. After double modification, more methacrylate groups were introduced in gelatin molecules. The hydrogels prepared from the double modified GelMAGMA macromer had higher crosslinking density and storage modulus than did the hydrogels prepared from single modified GelMA macromer. The GelMAGMA hydrogel was degraded more slowly than the GelMA hydrogel. By using the sequential double modification, gelatin hydrogels with a broad difference of storage modulus could be prepared.

Many methods have been reported to modulate the mechanical properties of gelatin hydrogels,

including the incorporation of nanoparticles, hybridization with other polymers, UV irradiation dosage and modification degree.[38,39] The previous two methods may induce other components in the hydrogels. High dosage and long UV irradiation may induce cellular damage. Modification degree can be modulated by binding a different amount of methacrylate groups with the amino groups in gelatin molecules. However, the range of mechanical properties that can be tuned by methacrylation of amino groups is limited. The sequential double modification of the amino, hydroxyl and carboxyl groups in gelatin broadened the tunable modification degree and mechanical property range. There are many crosslinking methods that can be used to prepare gelatin hydrogels and scaffolds for biomedical applications.[40-43] Crosslinking reagents, such as carbodiimides and succinimides,[43] glutaraldehyde,[44] and genipin,[45] and high energy irradiation,[46] have been used for the crosslinking. However, these methods should be used to crosslink the hydrogels and scaffolds before cell-lading to avoid cytotoxicity. In this study, UV-initiated polymerization was used to crosslink the GelMA and GelMAGMA macromers and had no evident negative influence on cell viability.

Both GelMA and GelMAGMA hydrogels showed promotive effects for chondrocytes to keep round morphology and express cartilaginous matrices. However, the hydrogels hampered cell proliferation. The effects should be due to the 3D microenvironment in hydrogels that are beneficial for chondrogenic differentiation, rather than cell proliferation. By comparison of the two gelatin hydrogels with low and high storage modulus, the GelMA hydrogel (low storage modulus) was more beneficial to cartilaginous matrices expression than the GelMAGMA hydrogel (high storage modulus). Physical cues, such as stiffness and cell size have been reported to have influences on cell functions.[47,48] In our previous study, gelatin hydrogel with the highest stiffness showed the best effects on maintaining the phenotype of chondrocytes. The gelatin hydrogel with the highest stiffness in the previous study was the GelMA hydrogel in this study. Further increasing the stiffness and storage modulus had no effect on the promotion of expression of cartilaginous matrices, nor the maintenance of chondrogenic phenotype. It has been reported that gelatin-hydroxyphenylpropionic acid hydrogel with a storage modulus of 1000 Pa has the best function for cartilage regeneration. Therefore, the GelMA hydrogel should provide the optimal microenvironment for maintenance of chondrocyte phenotypes. Influence of the second modification on the bioactive groups, such as the RGD motif in gelatin molecules might be another reason for the different effect on chondrocyte functions, which needs confirmation in the future. The good mechanical property and slow degradation of the GelMAGMA hydrogel should provide a variety of opportunities for incorporation of other bioactive factors or introduction of microporous structures in the hydrogels for cartilage tissue engineering.

3.5 Conclusions

Gelatin macromer with a high degree of modification was synthesized by sequential double modification of amino, hydroxyl and carboxyl groups in gelatin molecules. The double modified GelMAGMA macromer was used to prepare chondrocyte-laden hydrogel for 3D culturing of chondrocytes. The GelMAGMA hydrogel had a higher storage modulus and slower enzymatic degradation than did the hydrogel prepared with single modified GelMA macromer. The GelMAGMA hydrogel facilitated expression of cartilaginous matrices, as well as the maintenance of cartilage phenotype, while hampering cell proliferation. The GelMAGMA hydrogel should be useful for strengthening hydrogel scaffolds with a low degradation rate for cartilage tissue engineering.

3.6 References

1. Chen, G.; Sato, T.; Ushida, T.; Hirochika, R.; Tateishi, T. Redifferentiation of dedifferentiated bovine chondrocytes when cultured in vitro in a PLGA–collagen hybrid mesh. *FEBS Lett.* **2003**, *542*, 95-99.
2. Yan, S.; Zhang, X.; Zhang, K.; Di, H.; Feng, L.; Li, G.; Fang, J.; Cui, L.; Chen, X.; Yin, J. Injectable in situ forming poly (L-glutamic acid) hydrogels for cartilage tissue engineering. *J Mater Chem B* **2016**, *4*, 947-961.
3. Deepthi, S.; Gafoor, A.A.A.; Sivashanmugam, A.; Nair, S.V.; Jayakumar, R. Nanostrontium ranelate incorporated injectable hydrogel enhanced matrix production supporting chondrogenesis in vitro. *J Mater Chem B* **2016**, *4*, 4092-4103.
4. Wang, T.; Lai, J.H.; Han, L.-H.; Tong, X.; Yang, F. Modulating stem cell–chondrocyte interactions for cartilage repair using combinatorial extracellular matrix-containing hydrogels. *J Mater Chem B* **2016**, *4*, 7641-7650.
5. Serafim, A.; Tucureanu, C.; Petre, D.-G.; Dragusin, D.-M.; Salageanu, A.; Van Vlierberghe, S.; Dubruel, P.; Stancu, I.-C. One-pot synthesis of superabsorbent hybrid hydrogels based on methacrylamide gelatin and polyacrylamide. Effortless control of hydrogel properties through composition design. *New J. Chem.* **2014**, *38*, 3112-3126.
6. Zeng, L.; Chen, X.; Zhang, Q.; Yu, F.; Li, Y.; Yao, Y. Redifferentiation of dedifferentiated chondrocytes in a novel three‐dimensional microcavitary hydrogel. *J Biomed Mater Res A* **2015**, *103*, 1693-1702.
7. Nichol, J.W.; Koshy, S.T.; Bae, H.; Hwang, C.M.; Yamanlar, S.; Khademhosseini, A. Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials* **2010**, *31*, 5536-5544.
8. Yue, K.; Trujillo-de Santiago, G.; Alvarez, M.M.; Tamayol, A.; Annabi, N.; Khademhosseini, A. Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. *Biomaterials* **2015**, *73*, 254-271.
9. Li, X.; Chen, S.; Li, J.; Wang, X.; Zhang, J.; Kawazoe, N.; Chen, G. 3D culture of chondrocytes in gelatin hydrogels with different stiffness. *Polymers* **2016**, *8*, 269.
10. Zhang, J.; Yang, Y.; Chen, Y.; Liu, X.; Guo, S.; Zhu, L.; Wang, Y. An in situ phototriggered-imine-crosslink composite hydrogel for bone defect repair. *J Mater Chem B* **2016**, *4*, 973-981.
11. Yu, Y.; Guo, L.; Wang, W.; Wu, J.; Yuan, Z. Dual-peptide-modified alginate hydrogels for the promotion of angiogenesis. *Sci China Chem* **2015**, *58*, 1866-1874.
12. Han, L.; Xu, J.; Lu, X.; Gan, D.; Wang, Z.; Wang, K.; Zhang, H.; Yuan, H.; Weng, J. Biohybrid methacrylated gelatin/polyacrylamide hydrogels for cartilage repair. *J Mater Chem B* **2017**.
13. Wang, H.; Zhou, L.; Liao, J.; Tan, Y.; Ouyang, K.; Ning, C.; Ni, G.; Tan, G. Cell-laden photocrosslinked GelMA–DexMA copolymer hydrogels with tunable mechanical properties for tissue engineering. *J Mater Sci Mater Med* **2014**, *25*, 2173-2183.
14. Bryant, S.J.; Anseth, K.S. Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly (ethylene glycol) hydrogels. *J Biomed Mater Res A* **2002**, *59*, 63-72.

15. Metters, A.; Anseth, K.; Bowman, C. Fundamental studies of a novel, biodegradable PEG-b-PLA hydrogel. *Polymer* **2000**, *41*, 3993-4004.
16. Wang, L.-S.; Du, C.; Toh, W.S.; Wan, A.C.; Gao, S.J.; Kurisawa, M. Modulation of chondrocyte functions and stiffness-dependent cartilage repair using an injectable enzymatically crosslinked hydrogel with tunable mechanical properties. *Biomaterials* **2014**, *35*, 2207-2217.
17. Bian, L.; Hou, C.; Tous, E.; Rai, R.; Mauck, R.L.; Burdick, J.A. The influence of hyaluronic acid hydrogel crosslinking density and macromolecular diffusivity on human MSC chondrogenesis and hypertrophy. *Biomaterials* **2013**, *34*, 413-421.
18. Bartnikowski, M.; Bartnikowski, N.; Woodruff, M.; Schrobback, K.; Klein, T. Protective effects of reactive functional groups on chondrocytes in photocrosslinkable hydrogel systems. *Acta Biomater.* **2015**, *27*, 66-76.
19. Li, X.; Chen, Y.; Kawazoe, N.; Chen, G. Influence of microporous gelatin hydrogels on chondrocyte functions. *J Mater Chem B* **2017**.
20. Reis, A.V.; Fajardo, A.R.; Schuquel, I.T.; Guilherme, M.R.; Vidotti, G.J.; Rubira, A.F.; Muniz, E.C. Reaction of glycidyl methacrylate at the hydroxyl and carboxylic groups of poly (vinyl alcohol) and poly (acrylic acid): Is this reaction mechanism still unclear? *J. Org. Chem.* **2009**, *74*, 3750-3757.
21. Hoch, E.; Hirth, T.; Tovar, G.E.; Borchers, K. Chemical tailoring of gelatin to adjust its chemical and physical properties for functional bioprinting. *J Mater Chem B* **2013**, *1*, 5675-5685.
22. Li, X.; Li, B.; Ma, J.; Wang, X.; Zhang, S. Development of a silk fibroin/htcc/pva sponge for chronic wound dressing. *Journal of Bioactive and Compatible Polymers* **2014**, *29*, 398-411.
23. Chen, S.; Zhang, Q.; Nakamoto, T.; Kawazoe, N.; Chen, G. Gelatin scaffolds with controlled pore structure and mechanical property for cartilage tissue engineering. *Tissue Eng Part C Methods* **2016**, *22*, 189-198.
24. Cai, R.; Nakamoto, T.; Kawazoe, N.; Chen, G. Influence of stepwise chondrogenesis-mimicking 3D extracellular matrix on chondrogenic differentiation of mesenchymal stem cells. *Biomaterials* **2015**, *52*, 199-207.
25. Lu, H.; Ko, Y.-G.; Kawazoe, N.; Chen, G. Cartilage tissue engineering using funnel-like collagen sponges prepared with embossing ice particulate templates. *Biomaterials* **2010**, *31*, 5825-5835.
26. Chen, Y.C.; Lin, R.Z.; Qi, H.; Yang, Y.; Bae, H.; Melero‐Martin, J.M.; Khademhosseini, A. Functional human vascular network generated in photocrosslinkable gelatin methacrylate hydrogels. *Adv. Funct. Mater.* **2012**, *22*, 2027-2039.
27. Chen, S.; Zhang, Q.; Kawazoe, N.; Chen, G. Effect of high molecular weight hyaluronic acid on chondrocytes cultured in collagen/hyaluronic acid porous scaffolds. *RSC Adv.* **2015**, *5*, 94405-94410.
28. Fan, C.; Wang, D.A. Effects of permeability and living space on cell fate and neo‐tissue development in hydrogel‐based scaffolds: A study with cartilaginous model. *Macromol. Biosci.* **2015**, *15*, 535-545.
29. Kim, I.L.; Khetan, S.; Baker, B.M.; Chen, C.S.; Burdick, J.A. Fibrous hyaluronic acid hydrogels that direct MSC chondrogenesis through mechanical and adhesive cues. *Biomaterials* **2013**, *34*, 5571-5580.

30. Moreira Teixeira, L.; Leijten, J.; Sobral, J.; Jin, R.; Apeldoorn, A.; Feijen, J.; Blitterswijk, C.; Dijkstra, P.; Karperien, H. High throughput generated micro-aggregates of chondrocytes stimulate cartilage formation in vitro and in vivo. *Eur Cell Mater* **2012**, *23*, 387-399.
31. Fang, J.; Yong, Q.; Zhang, K.; Sun, W.; Yan, S.; Cui, L.; Yin, J. Novel injectable porous poly (γ-benzyl-l-glutamate) microspheres for cartilage tissue engineering: Preparation and evaluation. *J Mater Chem B* **2015**, *3*, 1020-1031.
32. Bryant, S.J.; Durand, K.L.; Anseth, K.S. Manipulations in hydrogel chemistry control photoencapsulated chondrocyte behavior and their extracellular matrix production. *J Biomed Mater Res A* **2003**, *67*, 1430-1436.
33. Gosset, M.; Berenbaum, F.; Thirion, S.; Jacques, C. Primary culture and phenotyping of murine chondrocytes. *Nat. Protoc.* **2008**, *3*, 1253.
34. Stewart, M.C.; Saunders, K.M.; Burton‐Wurster, N.; Macleod, J.N. Phenotypic stability of articular chondrocytes in vitro: The effects of culture models, bone morphogenetic protein 2, and serum supplementation. *J. Bone Miner. Res.* **2000**, *15*, 166-174.
35. Sung, L.-Y.; Lo, W.-H.; Chiu, H.-Y.; Chen, H.-C.; Chung, C.-K.; Lee, H.-P.; Hu, Y.-C. Modulation of chondrocyte phenotype via baculovirus-mediated growth factor expression. *Biomaterials* **2007**, *28*, 3437-3447.
36. De Crombrugghe, B.; Lefebvre, V.; Behringer, R.R.; Bi, W.; Murakami, S.; Huang, W. Transcriptional mechanisms of chondrocyte differentiation. *Matrix Biol.* **2000**, *19*, 389-394.
37. Lefrebvre, V.; de Crombrugghe, B. Toward understanding S0X9 function in chondrocyte differentiation. *Matrix Biol.* **1998**, *16*, 529-540.
38. Shin, S.R.; Aghaei‐Ghareh‐Bolagh, B.; Dang, T.T.; Topkaya, S.N.; Gao, X.; Yang, S.Y.; Jung, S.M.; Oh, J.H.; Dokmeci, M.R.; Tang, X.S. Cell‐laden microengineered and mechanically tunable hybrid hydrogels of gelatin and graphene oxide. *Adv. Mater.* **2013**, *25*, 6385-6391.
39. Miao, T.; Miller, E.J.; McKenzie, C.; Oldinski, R.A. Physically crosslinked polyvinyl alcohol and gelatin interpenetrating polymer network theta-gels for cartilage regeneration. *J Mater Chem B* **2015**, *3*, 9242-9249.
40. Zhang, J.; Li, J.; Chen, S.; Kawazoe, N.; Chen, G. Preparation of gelatin/Fe_3O_4 composite scaffolds for enhanced and repeatable cancer cell ablation. *J Mater Chem B* **2016**, *4*, 5664-5672.
41. Zhang, J.; Li, J.; Kawazoe, N.; Chen, G. Composite scaffolds of gelatin and gold nanoparticles with tunable size and shape for photothermal cancer therapy. *J Mater Chem B* **2017**, *5*, 245-253.
42. Truong, V.X.; Hun, M.L.; Li, F.; Chidgey, A.P.; Forsythe, J.S. In situ-forming click-crosslinked gelatin based hydrogels for 3D culture of thymic epithelial cells. *Biomater Sci* **2016**, *4*, 1123-1131.
43. Wissink, M.; Beernink, R.; Pieper, J.; Poot, A.A.; Engbers, G.; Beugeling, T.; Van Aken, W.; Feijen, J. Immobilization of heparin to EDC/NHS-crosslinked collagen. Characterization and in vitro evaluation. *Biomaterials* **2001**, *22*, 151-163.
44. Martucci, J.; Accareddu, A.; Ruseckaite, R. Preparation and characterization of plasticized gelatin films cross-linked with low concentrations of glutaraldehyde. *J Mater Sci* **2012**, *47*, 3282-3292.
45. Yan, L.P.; Wang, Y.J.; Ren, L.; Wu, G.; Caridade, S.G.; Fan, J.B.; Wang, L.Y.; Ji, P.H.;

Oliveira, J.M.; Oliveira, J.T. Genipin‐cross‐linked collagen/chitosan biomimetic scaffolds for articular cartilage tissue engineering applications. *J Biomed Mater Res A* **2010**, *95*, 465-475.

46. Zhang, X.; Xu, L.; Huang, X.; Wei, S.; Zhai, M. Structural study and preliminary biological evaluation on the collagen hydrogel crosslinked by γ‐irradiation. *J Biomed Mater Res A* **2012**, *100*, 2960-2969.
47. Higuchi, A.; Ling, Q.-D.; Kumar, S.S.; Chang, Y.; Alarfaj, A.A.; Munusamy, M.A.; Murugan, K.; Hsu, S.-T.; Umezawa, A. Physical cues of cell culture materials lead the direction of differentiation lineages of pluripotent stem cells. *J Mater Chem B* **2015**, *3*, 8032-8058.
48. Wang, X.; Nakamoto, T.; Dulińska-Molak, I.; Kawazoe, N.; Chen, G. Regulating the stemness of mesenchymal stem cells by tuning micropattern features. *J Mater Chem B* **2016**, *4*, 37-45.

Chapter 4

Influence of microporous gelatin hydrogels on chondrocyte functions

4.1 Summary

Hydrogels can provide biomimetic three-dimensional microenvironments for transplanted cells and are attractive scaffolds for cartilage tissue engineering. In this part, gelatin hydrogels with microporous structures were prepared and their effects on chondrocyte functions were compared with gelatin hydrogels without microporous structures. Gelatin bulk hydrogels were prepared by photo-initiated crosslinking of gelatin methacrylate macromers. Micropores were formed in the bulk hydrogels by dissolution of gelatin microgels prepared by a cutting method. Chondrocytes cultured in gelatin hydrogels without microporous structures showed high expression and production of cartilaginous matrices while low cell proliferation. Chondrocytes cultured in gelatin hydrogels with microporous structures tended to migrate from bulk hydrogel matrices to the micropores. Chondrocytes in the microporous hydrogels showed higher proliferation while lower expression and production of cartilaginous matrices than did the chondrocytes cultured in hydrogels without microporous structures. Gelatin hydrogels without microporous structures facilitated maintenance of cartilaginous phenotype of chondrocytes while microporous gelatin hydrogels were beneficial for cell proliferation.

4.2 Introduction

Cartilage tissue engineering has been developed as a useful approach for repairing of articular cartilage defects because articular cartilage has very limited regenerative capacity.[1-3] Tissue engineering approach usually combines cells, scaffolds and bioactive factors to induce regeneration of functional cartilage.[4-6] Scaffolds serve as a temporary support to accommodate cells and provide various signals for cell adhesion, proliferation, differentiation and secretion of extracellular matrices. A number of scaffolds have been

prepared from biodegradable polymers for cartilage tissue engineering.[7-10] Generally, biodegradable polymers are processed to form porous sponges or fabric scaffolds or hydrogels for applications in cartilage tissue engineering.

Although porous sponges or fabric scaffolds can provide plenty of vacant space to accommodate the transplanted cells, hydrogels have drawn extensive attention for cartilage tissue engineering due to their structural similarity to the microenvironments surrounding chondrocytes in vivo.[11-16] In cartilage tissue, chondrocytes are embedded in cartilaginous hydrogel-like matrices.[17] Hydrogels are physically or chemically crosslinked polymer networks that absorb plenty of water and swell in aqueous solution. Hydrogels have many advantages because they can provide a three-dimensional microenvironment by encapsulation of transplanted cells in the hydrogel matrices and can be delivered to defects by low-invasive injection. In hydrogels, transplanted cells are encapsulated in the hydrogel matrices that can provide similar in vivo microenvironments for cell differentiation and maintenance of phenotype.[18-21] Culture in hydrogels has been applied for chondrogenic differentiation of stem cells, such as bone-marrow derived mesenchymal stem cells and adipose derived mesenchymal stem cells. [19,20] They have also been used to maintain the phenotype of chondrocytes.[21] Hydrogels have shown better effects on chondrogenic differentiation than do porous sponges or fabric scaffolds. On the other hand, proliferation to obtain sufficient cell number is critical for cartilage tissue engineering because of the limited cell number from patients. Except for cell differentiation and maintenance of cell phenotype, hydrogels should provide favorable microenvironments to promote cell proliferation.

Introduction of micropores has been adopted to improve the microporous structures of hydrogels for accelerating cell proliferation.[22,23] To prepare microporous structures in hydrogels, gelatin, alginate and hyaluronic acid are usually used as porogen materials because they can be easily dissolved or degraded by thermal (body temperature), chemical or enzymatic treatments.[11] They have been used to prepare porous hydrogels of alginate, gelatin and fibrin.[23-26] The microporous structures promote cell migration, adhesion and proliferation. However, the porogen materials used to introduce microporous structures in hydrogels are usually different with the materials of the bulk hydrogel.[27] Although the porogen-forming materials are dissolved or degraded, most of them or their by-products are remained in the micropores and are gradually diffused through the bulk hydrogel matrices.[28] The micropores still contain some of the porogen-forming materials, which results in different microenvironments in the micropores and bulk hydrogel. To elucidate the effect of microstructures, the same porogen materials and bulk hydrogel matrices are desirable to eliminate the effect from the material difference.

Therefore, in this study, the same gelatin material was used for micropore formation and for bulk hydrogel preparation. Gelatin was used because gelatin has sol-gel translation temperature lower than body temperature and gelatin can be crosslinked by UV irradiation after methacrylation. Gelatin is also biocompatible and has been broadly used for cartilage tissue engineering. Gelatin microgels with microcubic shapes were prepared by a cutting method. Gelatin was sequentially modified with methacrylic anhydride and glycidyl methacrylate to prepare photocrosslinkable gelatin macromers. The gelatin microgels and photocrosslinkable gelatin macromers were mixed to prepare gelatin hydrogels with microporous structures. Gelatin hydrogels without microporous structures were used as a control. The behaviors of chondrocytes in the gelatin hydrogels with or without micropores were compared to disclose the effects of microporous structures on the chondrocyte functions.

4.3 Materials and methods

4.3.1 Synthesis of methacrylated gelatin macromers

Gelatin methacryloyl (GelMA) macromer was first synthesized according to a previously described method (Figure 4.1a).[29] Briefly, 5 g gelatin (type A, 300 bloom, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 50 mL phosphate buffered saline (PBS) at 60 ^{o}C under stirring to obtain a 10% (w/v) gelatin solution. 5 mL methacrylic anhydride (MA, Sigma-Aldrich, USA) was added into the gelatin solution at a rate of 0.5 mL/minutes under stirring at 50 ^{o}C. After the reaction was performed in dark for 3 hours, the resulting solution was diluted with 5-fold warm PBS (50 oC) and then was dialyzed against Milli-Q water at 40 ^{o}C for 7 days using a dialysis membrane (12-14 kD molecular weight cut-off, Spectrum Laboratories Inc. USA) to remove salts and excess free MA. After dialysis, GelMA macromer was obtained. The GelMA macromer was further modified with glycidyl methacrylate to prepare glycidyl methacrylate-modified GelMA (GelMAGMA macromer) by the following procedures (Figure 4.1a). The pH of GelMA macromer solution was adjusted to 3.5 and then 10 mL glycidyl methacrylate was added into the solution.[30] The reaction was conducted at 50 ^{o}C for 24 hours and then the product was dialyzed against milliQ water by the above-mentioned dialysis membrane at 40 ^{o}C for 7 days. The final products were lyophilized to obtain white foam and stored at -20 ^{o}C for further use.

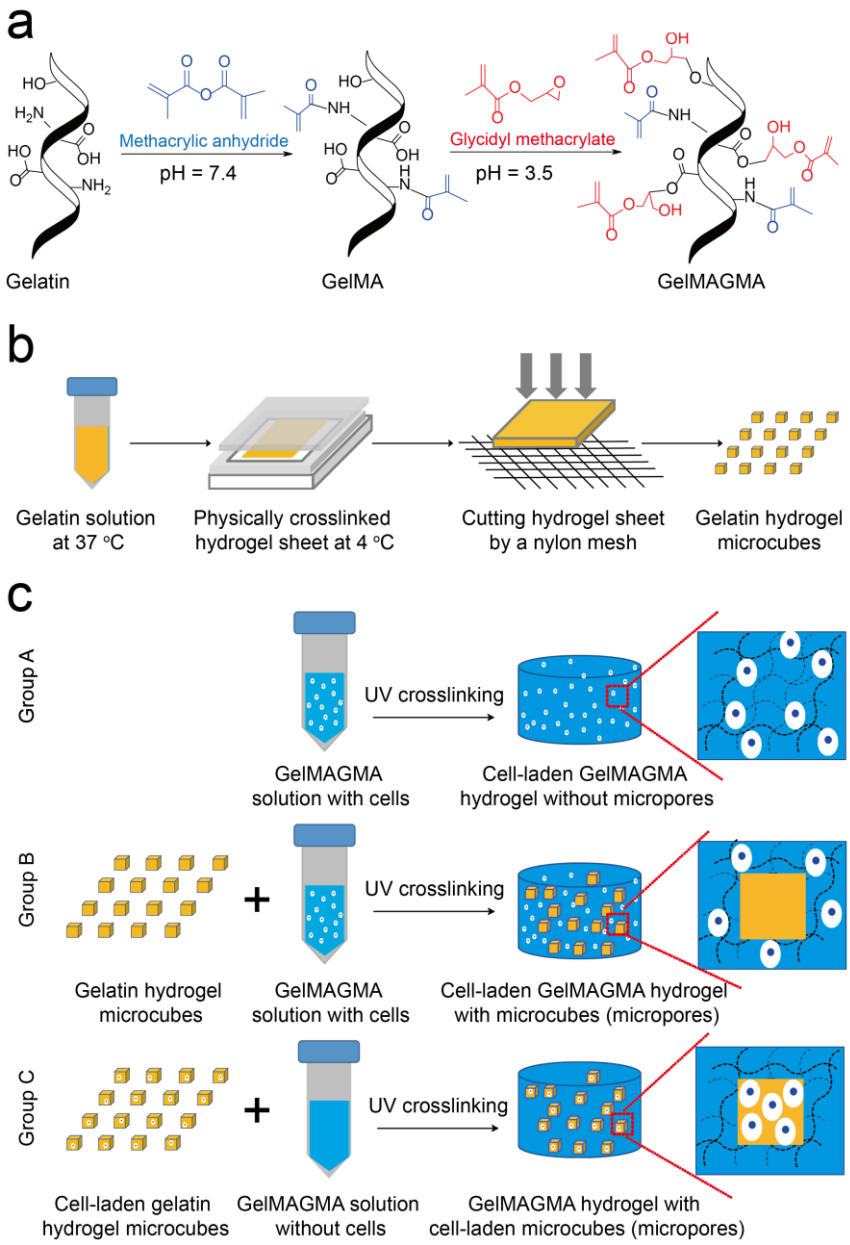

Figure 4.1 Preparation scheme of GelMAGMA macromers (a), mesh-cutted gelatin microgels (b) and GelMAGMA hydrogels with or without microporous structures (c).

4.3.2 ^{1}H nuclear magnetic resonance (NMR)

The degree of functionalization (DoF) of GelMA and GelMAGMA macromers were studied by using ^{1}H NMR according to a previously described method.[31] ^{1}H NMR spectra were collected by using a Varian INOVA NMR spectrometer with a single axis gradient inverse probe at a frequency of 300 MHz. Before the measurement, 20 mg of GelMA and GelMAGMA macromers were respectively dissolved into 1 mL deuterium oxide containing 0.05% (w/v) 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (Sigma-Aldrich, USA). The pristine gelatin without functionalization was also examined as a control.

4.3.3 Sol-gel translation property

MCR301 Rheometer (Anton Paar, Germany) was used to determine the rheological property of pristine gelatin, GelMA and GelMAGMA solutions (10% (w/v)). The solution was obtained by dissolving the powder or white foam into PBS at 50 ºC. 300 µL of each sample was used for the measurement. Oscillatory dynamic measurement was chosen using a parallel plate with the PP-50. Temperature sweep (from 37 ºC to 20 ºC, cooling rate was 0.15 ºC min^{-1}) was performed at a fixed amplitude (γ = 5%) and frequency (1 Hz). The sol-gel transition temperature was determined by the cross point of storage moduli (G') and loss moduli (G'').[31]

4.3.4 Chondrocytes isolation and subculture

Bovine articular chondrocytes (BACs) were isolated from articular cartilage from the knees of a 9 week-old calf according to previously reported protocol.[10] The isolated primary chondrocytes were cultured in 75 cm^2 tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 4500 mg L^{-1} glucose, 4 mM glutamine, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 1 mM sodium pyruvate and 50 µg mL^{-1} ascorbic acid at 37 ºC and 5% CO_2. The cell culture medium was refreshed every 3 days. The cells were subcultured after reaching confluence. Chondrocytes at passage 2 (P2) were used in the following experiments. Chondrocytes were detached with a trypsin/EDTA solution and collected by centrifugation. The chondrocytes were re-suspended in gelatin solution or GelMAGMA macromer solution in the following experiments.

4.3.5 Preparation of gelatin microcubes

Gelatin microgels with microcubic shapes were prepared by using a mesh-cutting method (Figure 4.1b). Gelatin solution (5% w/v) was prepared by dissolving pristine gelatin powder (type A, 175 bloom, Sigma-Aldrich) in a mixed solution that was prepared by mixing PBS and cell culture medium at a (v/v) ratio of 1:1. The gelatin solution was sterilized by filtration through a 0.22 µm filter. The sterilized gelatin solution was mixed with chondrocytes to prepare cell suspension solution at a cell concentration of 4×10^7 cells/mL. The cell suspension solution was poured into a sterile silicone frame having a space size of 100 mm × 20 mm × 300 µm that was placed on a sterile cover glass at 37 ºC. A sterile cover glass was placed on the silicone frame to control the thickness of solution. The glass plate/cell solution constructs were put in a 4 ºC refrigerator for 0.5 hour to allow gelation of cell suspension solution. The cell-laden gelatin hydrogel sheet was detached from the silicone frame and cover glasses and extended on a sterile nylon mesh having a mesh size of 250 µm × 250 µm (AS ONE, Japan). The cell-laden gelatin hydrogel sheet was gently pressed by a sterile spatula to pass through the nylon mesh. After the cell-laden gelatin hydrogel sheet was cut by the nylon mesh, cell-laden gelatin microgels having microcubic shapes were obtained. The cell-laden gelatin hydrogel microcubes were washed away from the mesh with sterile PBS and collected in 50 mL centrifuge tubes by centrifugation at 2000 rpm at 4 ºC. All the procedures were operated at sterile conditions. Gelatin hydrogel microcubes without chondrocytes were also prepared by the same procedures as described above by using gelatin solution without cells. The cell-laden gelatin hydrogel microcubes and pure gelatin hydrogel microcubes were used for following experiments.

4.3.6 Preparation of cell-laden GelMAGMA hydrogels without microporous structures

The GelMAGMA macromers were dissolved in PBS at 50 ^{o}C to prepare macromer aqueous solution at a concentration of 10 (w/v) %. Photo-initiator, 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (I 2959, Sigma-Aldrich, USA), was dissolved in the macromer aqueous solution at a concentration of 0.5 (w/v) % at 50 ^{o}C. The mixture solution was sterilized by filtration through a 0.22 μm filter and cooled down to room temperature. The sterilized mixture solution was mixed with the chondrocytes to prepare cell suspension solution in the GelMAGMA/photo-initiator mixture solution at a cell concentration of 2×10^7 cells/mL. The cell suspension solution was put between two quartz coverslips separated by a 1.5 mm-thick silicone spacer (KOKEN Co., Ltd., Tokyo, Japan) and exposed to UV light (CL-1000, Funakoshi Co., Ltd., Japan) at a distance of 20 cm for 5 minutes. After photo-initiated gelation, the cell-laden GelMAGMA hydrogels were punched into disks by a 6-mm biopsy punch (Group A in Figure 4.1c). The hydrogel disks had a diameter of 6 mm and a height of 1.5 mm. All the procedures were conducted under sterile conditions at room temperature. The cell-laden GelMAGMA hydrogel disks without gelatin hydrogel microcubes were put in T-flasks and cultured in DMEM at an atmosphere of 5% CO_2 at 37 ^{o}C with shaking.

4.3.7 Preparation of cell-laden GelMAGMA hydrogels with microporous structures

The above-prepared cell suspension solution in the GelMAGMA/photo-initiator mixture solution was added with pure gelatin hydrogel microcubes or cell-free GelMAGMA/photo-initiator mixture solution was added with cell-laden gelatin hydrogel microcubes at a mixture ratio of hydrogel microcubes and solution of 1: 2 (w/v) (Group B and C in Figure 4.1c). The mixture solution was put between the quartz coverslips, exposed to UV light and punched into disks having a diameter of 6 mm as above mentioned. The hydrogel disks were put in T-flasks and cultured in DMEM at an atmosphere of 5% CO_2 at 37 ^{o}C with shaking. They were cultured for 28 days and medium was changed every two days. All the procedures were conducted under sterile conditions at room temperature.

4.3.8 Cell viability assay

Live/dead staining was performed to evaluate in vitro cell viability of chondrocytes in the hydrogels by using Cellstain Double Staining Kit (Dojindo Laboratories, Tokyo, Japan). After UV crosslinking and 28 days of in vitro culture, the cell-laden GelMAGMA hydrogel disks with or without gelatin hydrogel microcubes were washed with PBS for three times and incubated with serum-free medium containing calcein-AM (2 μM) and propidium iodide (4 μM) at 37 ^{o}C for 15 minutes. The stained cells were observed using a confocal microscope (Zeiss LSM 510 Meta).

4.3.9 *In vivo* implantation

The animal experiment was approved by the animal experiments committee of the National Institute for Materials Science. All mice were maintained with a standard laboratory diet and water ad libitum. 4-week old athymic nude mice were used and all the operation of animal experiment was conducted according to the committee guidelines of the National Institute for Materials Science for Animal Experiments. After the

cell-laden GelMAGMA hydrogel disks with or without gelatin hydrogel microcubes were cultured in DMEM under an atmosphere of 5% CO_2 at 37 ºC with shaking for 1 day, the hydrogel disks (6 mm diameter × 1.5 mm height) were subcutaneously implanted into the dorsa of nude mice. Every mouse was implanted with four samples. After 12 weeks of implantation, the mice were euthanized and the samples were harvested for further investigation. The gross appearance of the implants was recorded using a digital camera.

4.3.10 Histological and immunohistochemical staining

After 12 weeks of implantation, the implants were carefully separated from the mice tissue, washed with PBS for three times and fixed with 10% neutral buffer formalin at room temperature for 2 days. The samples were dehydrated in a series of ethanol solution with increasing ethanol concentration from 70% to 99.5%, embedded in paraffin and sectioned to obtain cross-sections having 7 μm thickness. The cross-sections were deparaffinized, stained with hematoxylin and eosin (HE staining) for cell morphology and stained with alcian blue for glycosaminoglycan. Immunohistochemical staining of collagen type II was performed according to a previous report.[6] Briefly, the deparaffinized sections were incubated with proteinase K for 10 minutes for antigen retrieval and then incubated with peroxidase blocking solution for 5 minutes and 10% goat serum solution for 30 minutes. The sections were next incubated with the first antibody for 2 hours at room temperature, followed by incubation with the peroxidase labeled polymer-conjugated second antibody (DakoCytomation Envision+, Dako, Carpinteria, CA) for 30 minutes. The first antibody was rabbit polyclonal anti-bovine collagen type II at a 1:100 working dilution (Thermo Scientific, Rockford, IL). The sections were finally incubated with 3,3’-diaminobenzidine (DAB) for 10 minutes to develop color. The stained samples were observed under an optical microscope.

4.3.11 Quantification of DNA and sulfated glycosaminoglycan (sGAG)

DNA amount of cells in the hydrogels was quantified to evaluate the proliferation of chondrocytes. The cell-laden GelMAGMA hydrogels after in vitro culture for 2 and 4 weeks and after 12 weeks implantation were harvested, washed with PBS for three times and freeze-dried. The freeze-dried hydrogels and implants were digested by 500 μL papain solution (Sigma-Aldrich, USA), which was prepared by dissolving papain at a concentration of 400 mg/mL in 0.1 M PBS (pH 6.0) containing 5 mM cysteine hydrochloride and 5 mM ethylenediaminetetraacetic acid (EDTA). 5 μL of papain digestion solution was used to measure the DNA content with Hoechst 33258 dye (Sigma-Aldrich, USA). The fluorescence intensity was read with an FP-6500 spectrofluorometer (JASCO, Tokyo, Japan) at an excitation/emission wavelength of 360 nm and 460 nm. The sGAG content in each digestion solution was measured by using BlyscanTM Glycosaminoglycan Assay Kit (Biocolor Ltd., County Antrim, UK) according to the instructions offered by the products. Three samples in each group were used for the measurement to calculate means and standard deviations.

4.3.12 Real-time PCR analysis

Expression of genes encoding collagen type II and aggrecan genes was analyzed by a real-time polymerase chain reaction (PCR).[6] The implants after 12 weeks of implantation were harvested, washed with PBS for three times, frozen in liquid nitrogen and crushed into powder by an electric crusher. The

powder samples were dissolved in Sepasol solution (1 mL per sample) to isolate the RNA. The RNA content in each sample was quantified and then the RNA was converted to cDNA by MuLV reverse transcriptase (Applied Biosystems, USA). Real-time PCR was used to amplify glyceraldehyde-3-phosphate dehydrogenase (Gapdh), type II collagen (Col2a1) and aggrecan (Acan) by using a 7500 real-time PCR System (Applied Belgium, USA). The expression level of Gapdh, a housekeeping gene, was used as an endogenous control. The relative expression level of each target gene was calculated by using $2^{-\Delta\Delta Ct}$ formula and P2 chondrocytes as a reference. The primer and probe sequences were the same as the previous study.[10] Three samples in each group were used for the measurement to calculate means and standard deviations.

4.3.13 Statistical analysis

All data were reported as the mean ± standard deviation (SD). Statistical analysis was performed using a one-way analysis of variance and Tukey's post hoc test for multiple comparisons. All statistical analyses were executed by using KyPlot 2.0 beta 15.

4.4 Results and discussion

4.4.1 Synthesis and characterization of double modified gelatin macromer

GelMA macromer was synthesized by forming covalent bonds between the amino groups in gelatin molecules and methacrylic anhydride.[29] The GelMA macromer was further reacted with glycidyl methacrylate to synthesize GelMAGMA macromer (Figure 4.1a). The reaction was performed under acid condition (pH = 3.5) because it has been reported that the carboxyl and hydroxyl groups rather than amino groups react with glycidyl methacrylate through ring opening mechanism at acid environment.[30] The introduction of methacrylate groups in GelMA and GelMAGMA macromers was confirmed by ^{1}H NMR. The two obvious peaks at 5.4 and 5.7 ppm that were assigned to the vinyl protons of methacrylate groups were observed in the ^{1}H NMR spectrum of GelMA macromer. Furthermore, the peak intensity at $\delta = 2.9$ ppm that was assigned to the N-methylene protons in lysine groups decreased after reaction with methacrylic anhydride. After the second modification with glycidyl methacrylate, the peaks assigned to the vinyl protons of glycidyl methacrylate were observed at 5.8 and 6.2 ppm in the ^{1}H NMR of GelMAGMA macromer. The integrated intensity of the four peaks at 5.4, 5.7, 5.8 and 6.2 ppm in the ^{1}H NMR spectrum of the GelMAGMA macromer was almost two folds than that of the two peaks at 5.4 and 5.7 ppm in the ^{1}H NMR spectrum of GelMA macromer (Figure 4.2a). The results indicated the successful introduction of methacrylate and glycidyl methacrylate groups in the GelMAGMA macromer. In general, modification of gelatin with methacrylic anhydride or glycidyl methacrylate has been used for the preparation of gelatin hydrogels. In this study, methacrylic anhydride was used to react with amine groups in gelatin for first modification, and glycidyl methacrylate was used for second modification of carboxyl and hydroxyl groups in gelatin. The double modified gelatin should generate more crosslinkages during UV-initiated crosslinking reaction to form more stable hydrogels than the single modified gelatin macromers.

The rheological property of pristine gelatin, GelMA and GelMAGMA solution was investigated by oscillatory dynamic measurement. The G’ and G’’ of gelatin, GelMA and GelMAGMA solution at a concentration of 10 (w/v) % changed with temperature (Figure 4.2b). The sol-gel translation temperature was

measured from the cross point of G' exceeding G''.[31,32] The sol-gel translation temperature of pristine gelatin, GelMA and GelMAGMA was 36.0, 31.9 and 22.7 °C, respectively. The sol-gel translation temperature of GelMAGMA macromer was lower than room temperature. The decrease of sol-gel translation temperature of GelMA macromer should be due to a decrease of hydrogen atoms in amine groups and steric hindrance of methacrylate groups in GelMA for hydrogen bonding. Double modified GelMAGMA macromers further decreased the number of hydroxyl groups and carboxyl groups for hydrogen bonding.[31,33,34] The low translation temperature could maintain the GelMAGMA aqueous solution at a solution state at room temperature, which should allow easy cell suspension and incorporation of other materials, such as nano and micro particles in GelMAGMA macromer aqueous solution without heating.

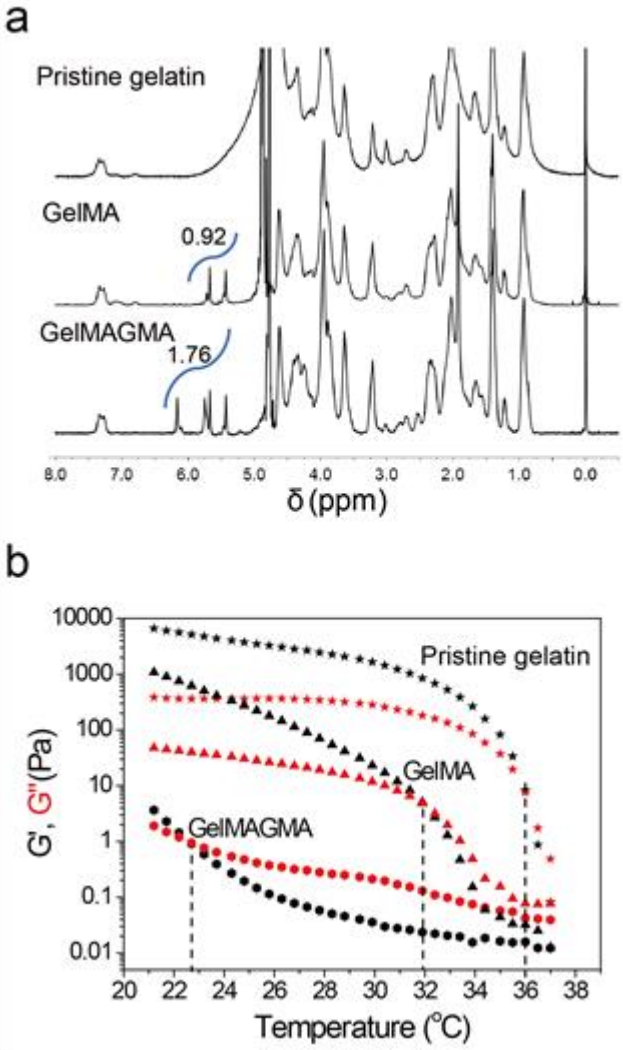

Figure 4.2 ^{1}H NMR spectra of pristine gelatin, GelMA and GelMAGMA macromers (a) and temperature-dependence curves of G' and G'' value of pristine gelatin, GelMA and GelMAGMA solution (10 %, w/v) (b).

4.4.2 Preparation of cell-laden GelMAGMA hydrogels with or without microporous structures

The GelMAGMA macromer was used to prepare hydrogels for 3D culture of chondrocytes. To

introduce microporous structure in the hydrogels, gelatin hydrogel microcubes were used because gelatin hydrogel microcubes could be prepared at a low temperature, such as 4 ^{o}C and dissolved at high temperature, such as 37 ^{o}C. The gelatin hydrogel microcubes were prepared by cutting gelatin hydrogel sheets with a nylon mesh (Figure 4.1b). The cutting method could avoid usage of cell-unfriendly organic solvents or edible oils that are usually used to prepare gelatin microbeads.[25] Preparation of gelatin hydrogel microcubes without usage of organic solvents or oils should be an attractive advantage of the method. Pure gelatin hydrogel microcubes and cell-laden gelatin hydrogel microcubes were prepared by the method and incorporated in the GelMAGMA hydrogels. The hydrogel microcubes with or without cells laden had the same dimension that was around 250 × 250 × 300 µm (Figure 4.3a).

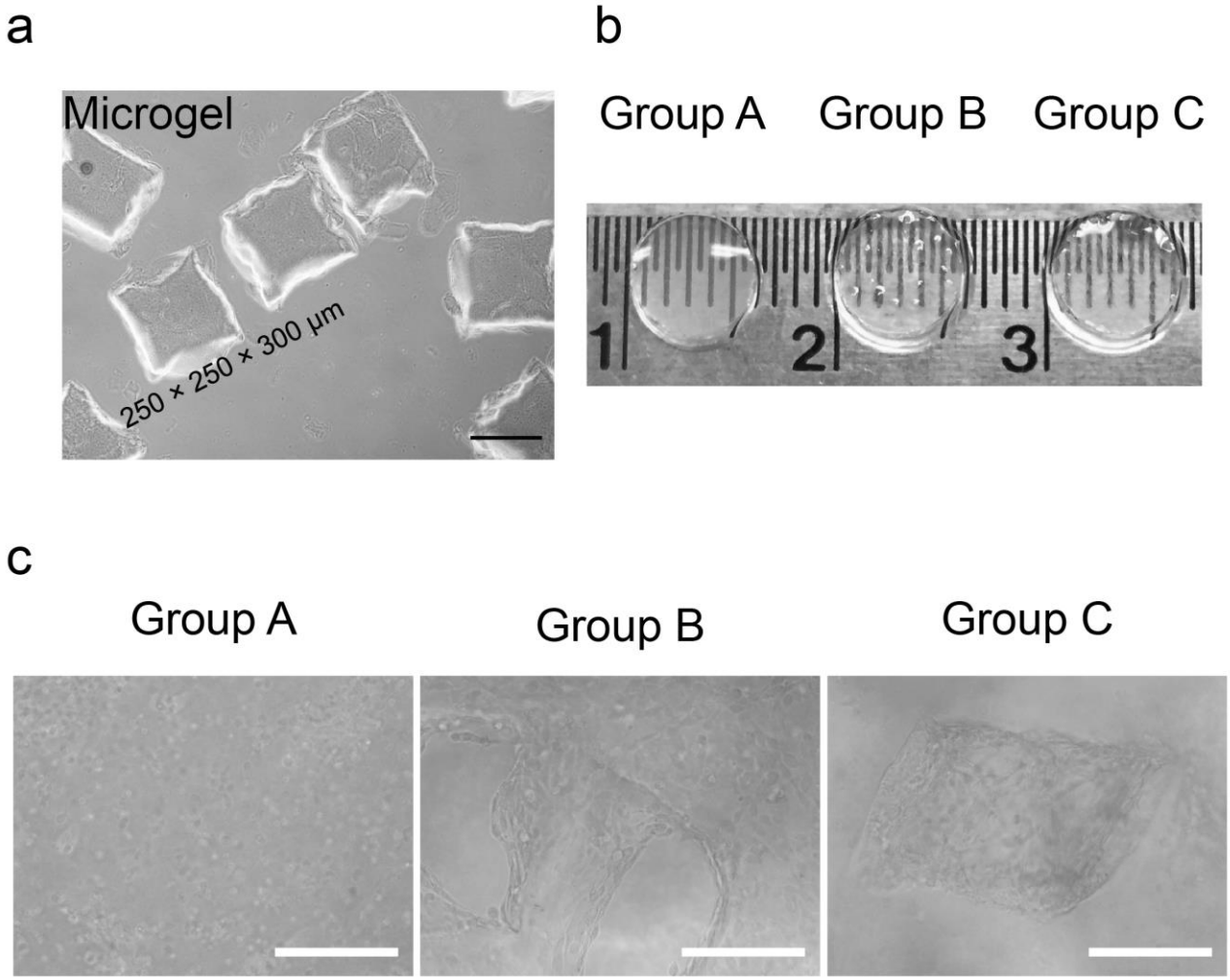

Figure 4.3 Photomicrographs of gelatin microgels prepared by a mesh-cutting method (a) and cell-laden GelMAGMA hydrogel without microporous structures (Group A), cell-laden GelMAGMA hydrogel with microporous structures where cells were seed in bulk hydrogel (Group B) or in micropores (Group C) (b). High magnification photomicrographs of Group A, B and C hydrogels after 7 days in vitro culture (c). Scale bar: 200 µm.

The cell-laden GelMAGMA hydrogels were prepared by using GelMAGMA macromer. GelMAGMA macromer was dissolved in PBS and the solution was mixed with chondrocytes. Furthermore, the gelatin hydrogel microcubes with or without chondrocytes were added to the mixture solution. The mixtures were irradiated by UV light to initiate crosslinking reaction to prepare GelMAGMA hydrogels. After crosslinking reaction, cell-laden GelMAGMA hydrogels were prepared. Three types of cell-laden GelMAGMA hydrogels were prepared by changing the preparation conditions (Table 4.1, Figure 4.1c). Group A was prepared from chondrocytes/GelMAGMA mixture solution without gelatin hydrogel microcubes. Group B was prepared from chondrocytes/GelMAGMA mixture solution with pure gelatin hydrogel microcubes. Group C was prepared from GelMAGMA solution with cell-laden gelatin hydrogel microcubes. All the three types of hydrogels were prepared from the same concentration of GelMAGMA macromer and had the same number of chondrocytes. The cell-laden GelMAGMA hydrogels were punched into hydrogel disks. The hydrogel disks had the same transparent gross appearance (Figure 4.3b).

The cell-laden GelMAGMA hydrogel disks were cultured in cell culture medium at 37 ºC. After culture for 7 days, they were observed by optical microscopy (Figure. 4.3c). Group A showed homogeneous hydrogel structure without any micropores available (Figure 4.3c). The cells were distributed homogeneously in the hydrogel matrices. Groups B and C had micropores in the hydrogels. Formation of the micropores in the hydrogels should be due to the dissolution of gelatin hydrogel microcubes at 37 ºC. Cell aggregates were observed at the peripheral edges of micropores in Group B. Cells showed even distribution in the micropores in Group C. The results indicated that chondrocytes in GelMAGMA hydrogel could migrate from hydrogel matrices to the micropores and predominately adhered and spread at the interface of micropores and medium (Group B). Chondrocytes seeded in the micropores did not migrate to the GelMAGMA hydrogel matrices (Group C).

Table 4.1 Preparation conditions of Group A, B and C hydrogels

Group	A	B	C
GelMAGMA solution	2 mL (4×10^7 cells)	2 mL (4×10^7 cells)	2 mL (no cell)
Microgel		1 g (no cell)	1 g (4×10^7 cells)

4.4.3 Cell viability and proliferation in GelMAGMA hydrogels during *in vitro* culture

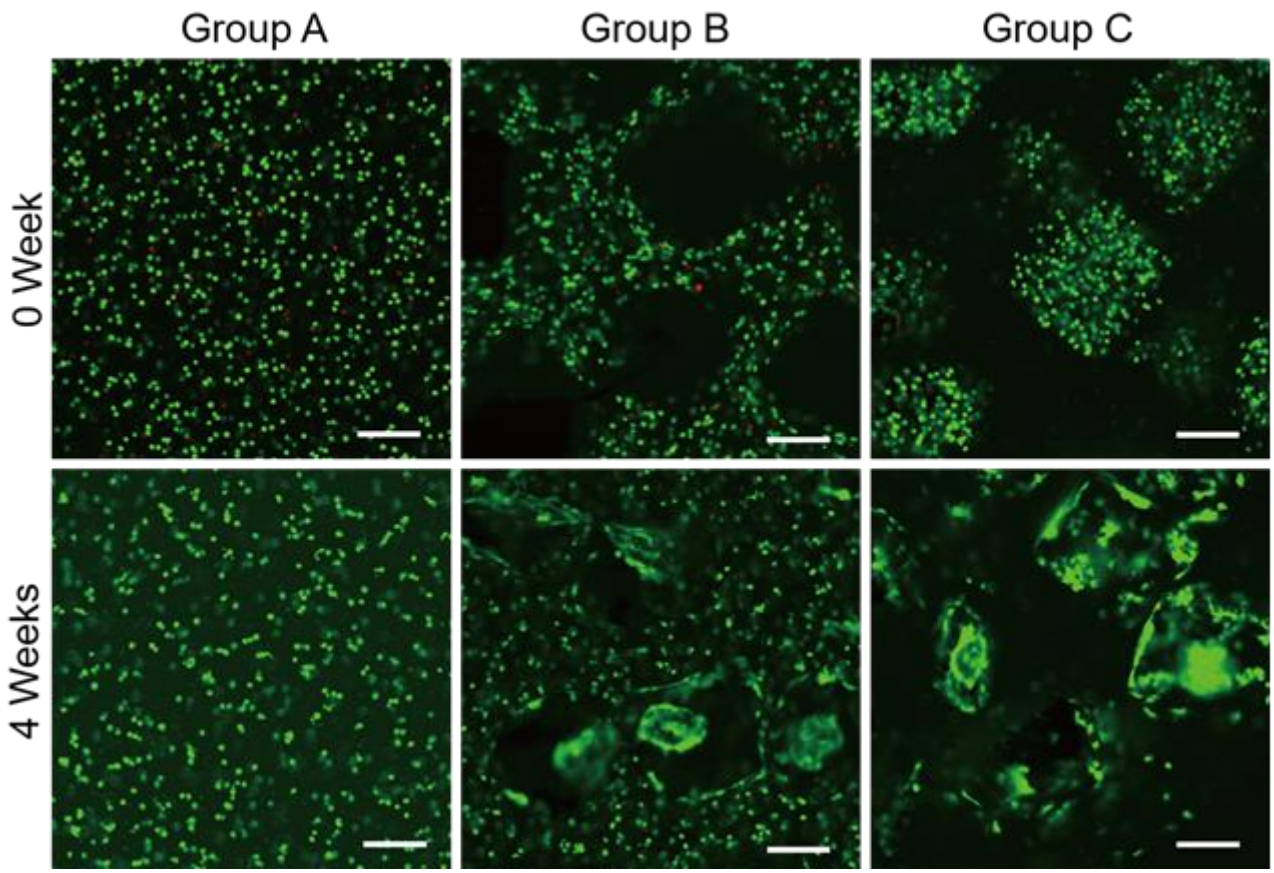

Figure 4.4 Live and dead staining of chondrocytes in the hydrogels immediately after UV crosslinking (0 week) and after 28 days *in vitro* culture (4 weeks). Scale bar: 200 µm.

After four weeks of in vitro culture, no obvious degradation or deformation of GelMAGMA hydrogel disks was observed in any of the three groups. Cell distribution and viability in the GelMAGMA hydrogels immediately after UV crosslinking and after 4 weeks culture were examined by live/dead staining (Figure 4.4). After UV crosslinking (day 0), only a very small amount of dead cells (stained red) were observed in Group A and B. Few dead cells were observed in Group C. The results suggested that UV crosslinking process did not have an obvious effect on cell viability. Cells were distributed homogeneously in Group A. In Group B, cells in the crosslinked GelMAGMA bulk hydrogel matrices showed homogeneous distribution while no cells were detected in the pure gelatin hydrogel microcubes. In Group C, cells were homogeneously distributed only in the cell-laden gelatin hydrogel microcubes while no cells were detected in the crosslinked GelMAGMA bulk hydrogel matrices, which was opposite to that in Group B. Homogeneous cell distribution in Group A should be due to the even suspension of chondrocytes in the GelMAGMA solution before UV irradiation. Cells were only observed in the crosslinked GelMAGMA bulk hydrogel matrices in Group B because cells were only suspended in the GelMAGMA solution and cell-free gelatin hydrogel microcubes were used for the preparation of Group B. Cells were only observed in the gelatin hydrogel microcubes in Group C was because cell-laden gelatin hydrogel microcubes and cell-free GelMAGMA solution were used. The gelatin hydrogel microcubes maintained their cubic shape immediately after UV crosslinking reaction and then were gradually dissolved during cell culture.

After in vitro culture for 4 weeks, chondrocytes in all the groups showed high cell viability. Cell distribution was different from that at the beginning. Cell aggregates were observed in all the three groups and cell aggregates were larger in Group B and C than those in Group A. Cell density seemed less dense than that at the beginning, which should be due to cell aggregation. Cells were still homogeneously distributed in Group A and cell aggregates were small. However, cell distribution in Group B and C showed obvious change compared to that observed at the beginning. In Group B, big cell aggregates were observed in the micropores while relatively homogeneous cell distribution was observed in the GelMAGMA bulk hydrogel matrices. In Group C, big cell aggregates were observed in the micropores while no cells were observed in the GelMAGMA bulk hydrogel matrices. The results indicated that some of the cells seeded in the GelMAGMA bulk hydrogel migrated to the micropores and proliferated to form big aggregates in Group B. The cells seeded in the micropores in Group C resided in the micropores and proliferated to form big aggregates without migration to the GelMAGMA bulk hydrogel matrices.

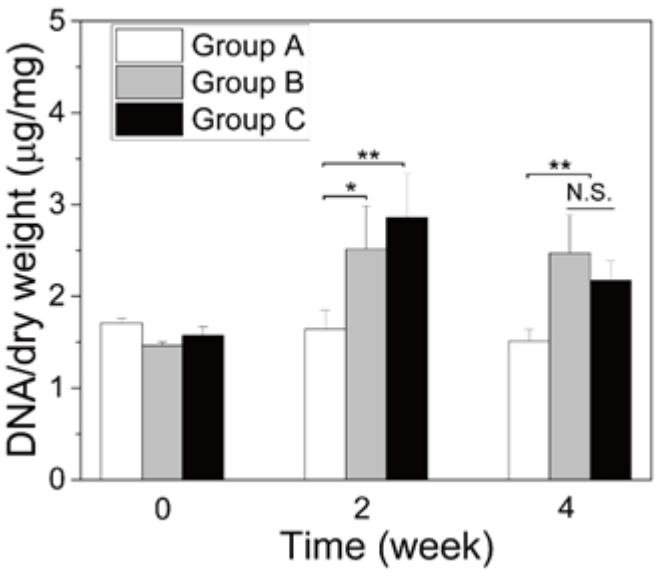

Figure 4.5 Quantification of DNA amount in the Group A, B and C hydrogels during *in vitro* culture. The data were normalized with dry weight of the hydrogels. Means ± SD, N=3. *, $p < 0.05$; and **, $p < 0.01$.

Quantification of DNA amount showed that DNA amount in Group A during 4 weeks in vitro culture did not change (Figure 4.5). DNA amount in Group B and C increased during the first 2 weeks of in vitro culture and did not change (Group B) or decreased a little (Group C) during the next 2 weeks of in vitro culture. The results indicated that chondrocytes in the GelMAGMA bulk hydrogel had high viability but did no proliferate. Increase of DNA amount in Group B for the first 2 weeks should be due to the proliferation of migrated chondrocytes in the micropores. The micropores formed from dissolution of gelatin hydrogel microcubes allowed the adhesion and proliferation of migrated chondrocytes. The micropores in Group B and C provided vacant spaces for cell proliferation at the first two weeks. Nutrient diffusion limitation of the GelMAGMA bulk after 2 weeks culture might hamper further cell proliferation.

4.4.4 Histological and immunohistochemical staining of *in vivo* implants

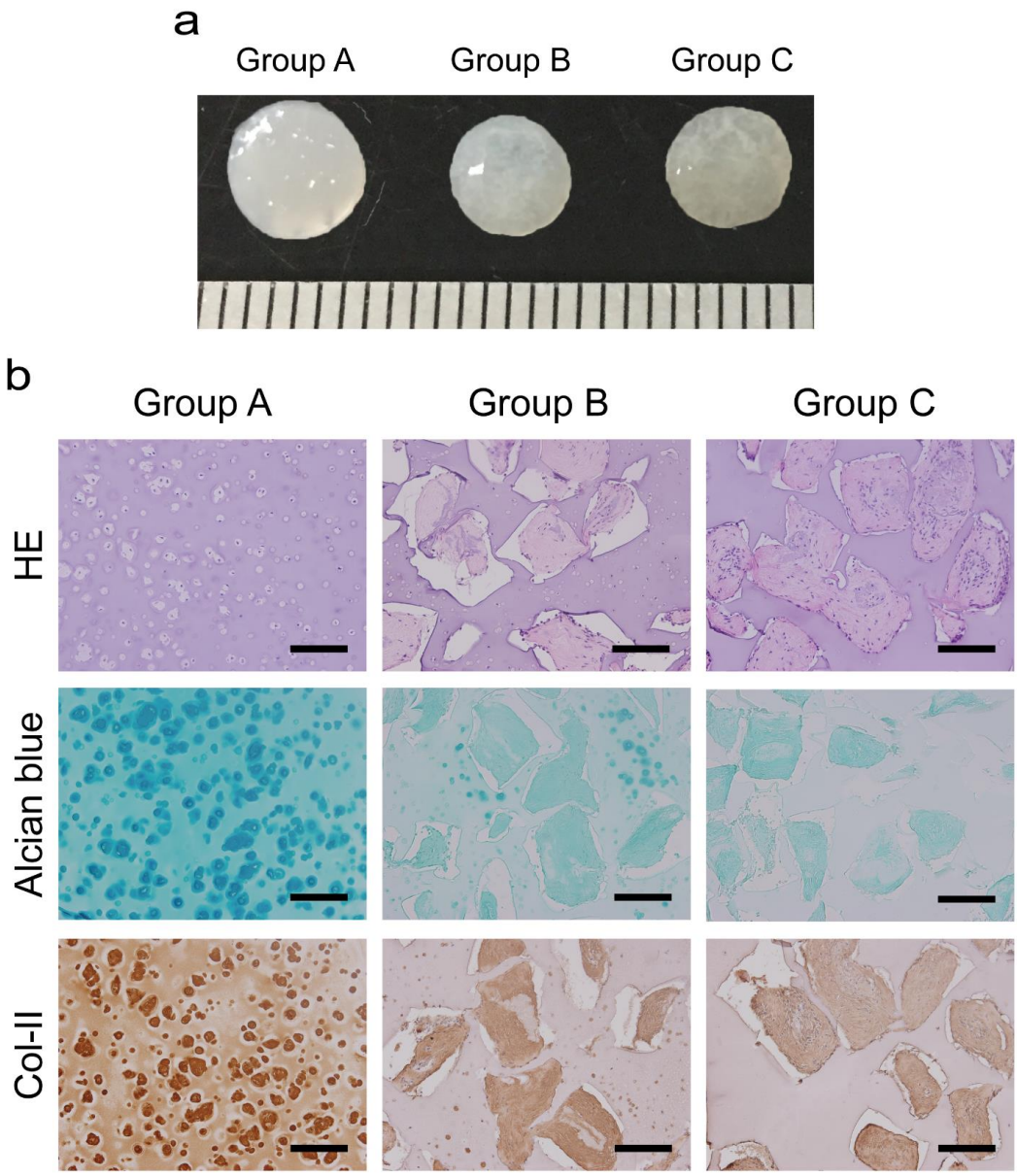

Figure 4.6 Gross appearance of Group A, B and C implants after 12 weeks implantation (a). Representative images of hematoxylin and eosin (HE) staining, alcian blue (Alcian blue) staining and immunohistochemical staining of type II collagen (Col-II) of Group A, B and C implants after 12 weeks

The GelMAGMA hydrogel disks were implanted subcutaneously in nude mice for 12 weeks. All the implants showed glistening white gross appearance (Figure 4.6a). The GelMAGMA hydrogel implants with microporous structures (Groups B and C) were slightly smaller than the GelMAGMA hydrogel implants without microporous structures, which indicated slight shrinkage of the GelMAGMA hydrogel implants with microporous structures. The shrinkage should be due to the microporous structures and cell-induced shrinkage in the hydrogels.[35]

Cell morphology, distribution and extracellular matrices (ECMs) in the implants were examined by histological and immunohistochemical staining (Figure 4.6b). HE staining showed that chondrocytes in Group A had round morphology and distributed homogeneously in the matrices. Chondrocytes in Groups B

and C had a heterogeneous distribution. Cell density in the micropores was very high while cell density in GelMAGMA bulk hydrogel matrices was very low. In particular, in Group C no cells were observed in GelMAGMA bulk hydrogel matrices. Alcian blue staining showed that there was abundance of cartilaginous matrices in Group A implants. Most of the cartilaginous matrices were detected surrounding the chondrocytes. In Group B implants, extracellular matrices in micropores were very positively stained while the extracellular matrices in GelMAGMA bulk hydrogel were slightly stained. In Group C implants, only the extracellular matrices in micropores were stained by alcian blue. The GelMAGMA bulk hydrogel matrices were not positively stained. Immunohistochemical staining of type II collagen showed that type II collagen was detected surrounding chondrocytes in Group A, distributing throughout the implant. However, in Group B, type II collagen was predominately detected in micropores and slightly in GelMAGMA bulk hydrogel matrices. In Group C, type II collagen was only detected in micropores, not in the GelMAGMA bulk hydrogel matrices. The immunohistochemical staining results of type II collagen were similar to that of alcian blue staining results. The results indicated that cartilaginous matrices were homogeneously distributed in hydrogels without microporous structures while predominately distributed in the micropores of microporous hydrogels.

4.4.5 DNA and sGAG quantification and cartilaginous gene expression of *in vivo* implants

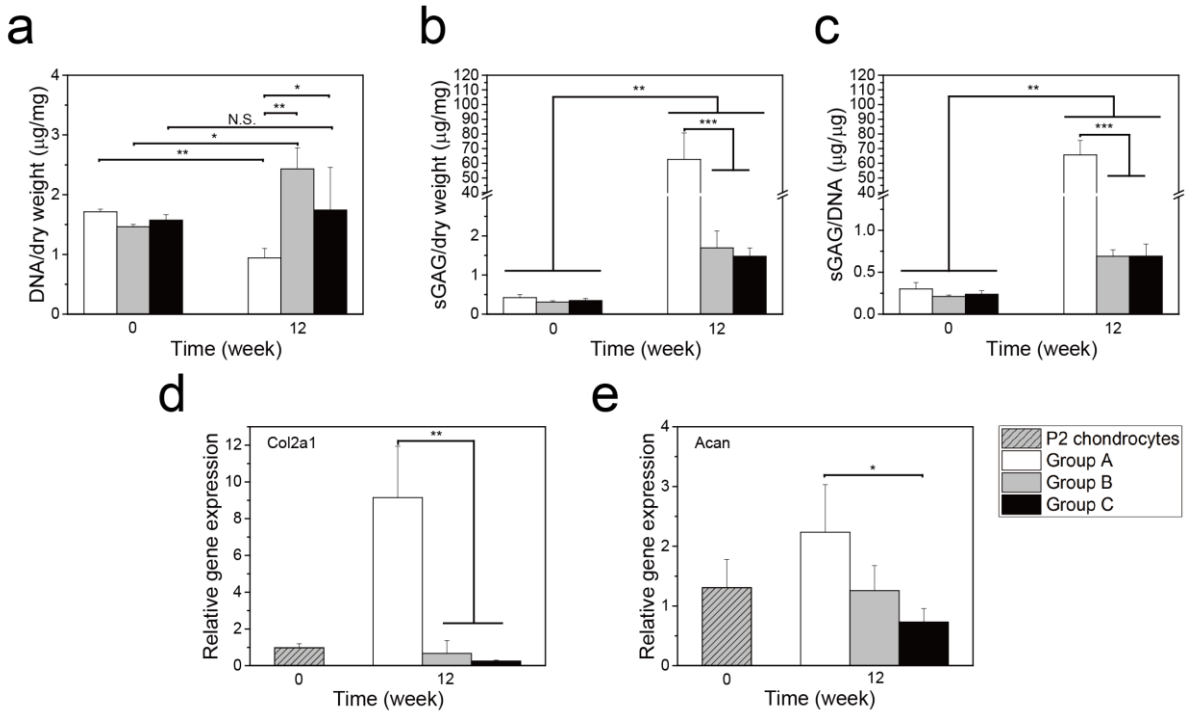

Figure 4.7 Quantification of DNA amount (a) and sGAG content (b) in the implants after 12 weeks implantation. The data were normalized with dry weight of the hydrogels. sGAG/DNA ratio in each implant after 12 weeks implantation (c). Expression of genes encoding collagen type II (d) and aggrecan (e) of P2 chondrocytes used for cell seeding (0 week) and chondrocytes in the implants after 12 weeks implantation. Gene expression data were normalized by using P2 chondrocytes as a reference. Means ± SD, N=3. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

DNA amount and sGAG content in the implants before implantation and after 12 weeks implantation were quantified (Figure 4.7a). After 12 weeks of in vivo implantation, Group B implants had the highest DNA amount while Group A implants had the lowest. Compared to the DNA amount before implantation, DNA amount in Group A decreased, in Group C kept at the same level while in Group B increased. The results indicated that chondrocytes proliferated only in Group B, which might be due to the proliferation of migrated chondrocytes in the micropores of Group B. In vitro culture results also indicated that the chondrocytes seeded in GelMAGMA bulk hydrogel migrated to the micropores, adhered and spread at the micropore interface. The migrated chondrocytes in the micropores in Group B proliferated during in vitro culture. Decrease of DNA amount in Group A implants might be due to partial cell death during long period implantation. Formation of cartilaginous matrices in the GelMAGMA bulk hydrogel might hamper diffusion of nutrients and therefore affect cell proliferation. Chondrocytes in Group C did not proliferate during implantation. The porous structures in Group C implants should improve nutrient diffusion that was important for chondrocytes proliferation while high cell density in the micropores at the beginning could form aggregates and therefore hamper cell proliferation. Cell behaviors in Group C should be the sum of the contrary effects of microporous structure and high cell seeding density.

The sGAG content and sGAG/DNA value in all the groups increased significantly after 12 weeks implantation (Figure 4.7b, 4.7c). After 12 weeks implantation, the sGAG content and sGAG/DNA value in Group A implants were significantly higher than those in Group B and C implants. The sGAG content and sGAG/DNA value in Group B and C were at the same level. The results indicated that gelatin hydrogel without microporous structures could promote the production of cartilaginous matrices more strongly than did gelatin hydrogels with microporous structures. Gene expression of type II collagen and aggrecan indicated that chondrocytes in Group A implants showed the highest expression of these two cartilaginous genes (Figure 4.7d and 4.7e). Expression of type II collagen and aggrecan in Group A implants was promoted while that in Group B and C did not change or decreased a little.

The results from both in vitro cell culture and in vivo implantation showed that chondrocytes showed different behaviors in Group A, B and C hydrogels. In GelMAGMA hydrogels without microporous structures (Group A), chondrocytes were distributed homogeneously in hydrogel matrices, formed small cell aggregates and showed high production and expression of cartilaginous matrices without proliferation. On the other hand, in GelMAGMA hydrogels with microporous structures (Group B and C), chondrocytes were predominantly distributed in the micropores, spread and proliferated with low production and expression of cartilaginous matrices. In gelatin hydrogels with microporous structures, cell seeding method also affected cell functions. Cell seeded in the bulk hydrogels (Group B) partially migrated in the micropores, adhered and spread at the interfaces of micropores and medium. The migrated chondrocytes could proliferate quickly in the micropores to fill the spaces and meanwhile to form big cell aggregates. Cell seeding in the micropores (Group C) constrained all the chondrocytes in the micropores without migration to bulk hydrogels, which resulted in high cell density in the micropores. The high density of chondrocytes in the micropores could induce the formation of big cell aggregates with very limited proliferation.[36,37]

Comparison of gelatin hydrogels with or without microporous structures indicated that culture in gelatin hydrogels without microporous structures was favorable for the production of cartilaginous matrices but not for cell proliferation, while culture in gelatin hydrogels with microporous structures was beneficial for cell proliferation but not for the production of cartilaginous matrices. Gelatin hydrogels without microporous structures could provide three-dimensional microenvironments that were similar to the in vivo microenvironment which is favorable to cell differentiation. It has been reported that three-dimensional microenvironments promote chondrogenic differentiation of stem cells or maintain the chondrogenic

morphology of chondrocytes.[18-21] Pore structures in porous sponges or fabric scaffolds have been reported to facilitate cell proliferation.[38] The pores in porous sponges or fabric scaffolds should not only provide spaces for accommodation, adhesion and proliferation of seeded cells but also provide interconnected microporous structures for cell migration and nutrient diffusion in the scaffolds. The introduction of microporous structures in hydrogels has been proposed and many microporous hydrogels have been reported for tissue engineering of a variety of tissues. However, cell proliferation in microporous hydrogels is slower than that in porous sponges or fabric scaffolds, which should be due to the difference of pore interconnectivity.[8] Pore interconnectivity should be considered to further improve the functions of microporous hydrogels for tissue engineering applications.

4.5 Conclusions

Chondrocyte-laden gelatin hydrogels with or without microporous structures were prepared with photocrosslinkable gelatin methacrylate macromer and gelatin microgels. The gelatin microgels with microcubic shapes were dissolved to form micropores in the hydrogels during cell culture. Chondrocytes were encapsulated in the gelatin bulk hydrogels or released from the micropore-forming gelatin microgels. Effects of microporous structures on the proliferation and differentiation of chondrocytes were disclosed. Chondrocytes migrated from gelatin bulk hydrogels to the micropores while not from the micropores to the bulk hydrogels. Gelatin hydrogels without microporous structures promoted expression and production of cartilaginous matrices while hampered cell proliferation. Gelatin hydrogels with microporous structures were beneficial for cell proliferation while had not evident effect on the expression and production of cartilaginous matrices. Gelatin hydrogels with different microporous structures showed different effects on chondrocyte functions. The results should provide useful information for preparation of scaffolds for tissue engineering.

4.6 References

1. Huang, B.J.; Hu, J.C.; Athanasiou, K.A. Cell-based tissue engineering strategies used in the clinical repair of articular cartilage. *Biomaterials* **2016**, *98*, 1-22.
2. Lu, H.; Ko, Y.-G.; Kawazoe, N.; Chen, G. Cartilage tissue engineering using funnel-like collagen sponges prepared with embossing ice particulate templates. *Biomaterials* **2010**, *31*, 5825-5835.
3. He, X.; Lu, H.; Kawazoe, N.; Tateishi, T.; Chen, G. A novel cylinder-type poly (L-lactic acid)–collagen hybrid sponge for cartilage tissue engineering. *Tissue Eng Part C Methods* **2009**, *16*, 329-338.
4. Moutos, F.T.; Freed, L.E.; Guilak, F. A biomimetic three-dimensional woven composite scaffold for functional tissue engineering of cartilage. *Nat. Mater.* **2007**, *6*, 162-167.
5. Chen, S.; Zhang, Q.; Nakamoto, T.; Kawazoe, N.; Chen, G. Highly active porous scaffolds of collagen and hyaluronic acid prepared by suppression of polyion complex formation. *J Mater Chem B* **2014**, *2*, 5612-5619.
6. Cai, R.; Nakamoto, T.; Kawazoe, N.; Chen, G. Influence of stepwise chondrogenesis-mimicking 3D extracellular matrix on chondrogenic differentiation of mesenchymal stem cells. *Biomaterials* **2015**, *52*, 199-207.
7. Hsu, S.-h.; Hung, K.-C.; Chen, C.-W. Biodegradable polymer scaffolds. *J Mater Chem B*

2016, *4*, 7493-7505.

8. Chen, S.; Zhang, Q.; Nakamoto, T.; Kawazoe, N.; Chen, G. Gelatin scaffolds with controlled pore structure and mechanical property for cartilage tissue engineering. *Tissue Eng Part C Methods* **2016**, *22*, 189-198.
9. Chen, S.; Zhang, Q.; Kawazoe, N.; Chen, G. Effect of high molecular weight hyaluronic acid on chondrocytes cultured in collagen/hyaluronic acid porous scaffolds. *RSC Adv.* **2015**, *5*, 94405-94410.
10. Zhang, Q.; Lu, H.; Kawazoe, N.; Chen, G. Pore size effect of collagen scaffolds on cartilage regeneration. *Acta Biomater.* **2014**, *10*, 2005-2013.
11. Vega, S.; Kwon, M.; Burdick, J. Recent advances in hydrogels for cartilage tissue engineering. *Eur Cell Mater* **2017**, *33*, 59-75.
12. Han, L.; Xu, J.L.; Lu, X.; Gan, D.L.; Wang, Z.X.; Wang, K.F.; Zhang, H.P.; Yuan, H.P.; Weng, J. Biohybrid methacrylated gelatin/polyacrylamide hydrogels for cartilage repair. *J Mater Chem B* **2017**, *5*, 731-741.
13. Zhang, K.; He, S.; Yan, S.; Li, G.; Zhang, D.; Cui, L.; Yin, J. Regeneration of hyaline-like cartilage and subchondral bone simultaneously by poly (L-glutamic acid) based osteochondral scaffolds with induced autologous adipose derived stem cells. *J Mater Chem B* **2016**, *4*, 2628-2645.
14. Cao, L.; Cao, B.; Lu, C.; Wang, G.; Yu, L.; Ding, J. An injectable hydrogel formed by in situ cross-linking of glycol chitosan and multi-benzaldehyde functionalized PEG analogues for cartilage tissue engineering. *J Mater Chem B* **2015**, *3*, 1268-1280.
15. Hunt, J.A.; Chen, R.; van Veen, T.; Bryan, N. Hydrogels for tissue engineering and regenerative medicine. *J Mater Chem B* **2014**, *2*, 5319-5338.
16. Spiller, K.L.; Maher, S.A.; Lowman, A.M. Hydrogels for the repair of articular cartilage defects. *Tissue Eng Part B Rev.* **2011**, *17*, 281-299.
17. Reddi, A.H.; Becerra, J.; Andrades, J.A. Nanomaterials and hydrogel scaffolds for articular cartilage regeneration. *Tissue Eng Part B Rev.* **2011**, *17*, 301-305.
18. Chung, C.; Burdick, J.A. Influence of three-dimensional hyaluronic acid microenvironments on mesenchymal stem cell chondrogenesis. *Tissue Eng. Part A* **2008**, *15*, 243-254.
19. Aisenbrey, E.; Bryant, S. Mechanical loading inhibits hypertrophy in chondrogenically differentiating hMSCs within a biomimetic hydrogel. *J Mater Chem B* **2016**, *4*, 3562-3574.
20. Awad, H.A.; Wickham, M.Q.; Leddy, H.A.; Gimble, J.M.; Guilak, F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials* **2004**, *25*, 3211-3222.
21. Callahan, L.A.S.; Ganios, A.M.; Childers, E.P.; Weiner, S.D.; Becker, M.L. Primary human chondrocyte extracellular matrix formation and phenotype maintenance using RGD-derivatized PEGDM hydrogels possessing a continuous young's modulus gradient. *Acta Biomater.* **2013**, *9*, 6095-6104.
22. Chuah, Y.J.; Peck, Y.; Lau, J.E.J.; Hee, H.T.; Wang, D.-A. Hydrogel based cartilaginous tissue regeneration: Recent insights and technologies. *Biomater Sci* **2017**, *5*, 613-631.
23. Han, L.-H.; Lai, J.H.; Yu, S.; Yang, F. Dynamic tissue engineering scaffolds with stimuli-responsive macroporosity formation. *Biomaterials* **2013**, *34*, 4251-4258.
24. Zeng, L.; Chen, X.; Zhang, Q.; Yu, F.; Li, Y.; Yao, Y. Redifferentiation of dedifferentiated chondrocytes in a novel three‐dimensional microcavitary hydrogel. *J Biomed Mater Res A*

2015, *103*, 1693-1702.

25. Leong, W.; Lau, T.T.; Wang, D.-A. A temperature-cured dissolvable gelatin microsphere-based cell carrier for chondrocyte delivery in a hydrogel scaffolding system. *Acta Biomater.* **2013**, *9*, 6459-6467.
26. Liu, J.; Xu, H.H.; Zhou, H.; Weir, M.D.; Chen, Q.; Trotman, C.A. Human umbilical cord stem cell encapsulation in novel macroporous and injectable fibrin for muscle tissue engineering. *Acta Biomater.* **2013**, *9*, 4688-4697.
27. Sun, J.; Wei, D.; Zhu, Y.; Zhong, M.; Zuo, Y.; Fan, H.; Zhang, X. A spatial patternable macroporous hydrogel with cell-affinity domains to enhance cell spreading and differentiation. *Biomaterials* **2014**, *35*, 4759-4768.
28. Huebsch, N.; Lippens, E.; Lee, K.; Mehta, M.; Koshy, S.T.; Darnell, M.C.; Desai, R.M.; Madl, C.M.; Xu, M.; Zhao, X. Matrix elasticity of void-forming hydrogels controls transplanted-stem-cell-mediated bone formation. *Nat. Mater.* **2015**, *14*, 1269-1277.
29. Li, X.; Chen, S.; Li, J.; Wang, X.; Zhang, J.; Kawazoe, N.; Chen, G. 3D culture of chondrocytes in gelatin hydrogels with different stiffness. *Polymers* **2016**, *8*, 269.
30. Reis, A.V.; Fajardo, A.R.; Schuquel, I.T.; Guilherme, M.R.; Vidotti, G.J.; Rubira, A.F.; Muniz, E.C. Reaction of glycidyl methacrylate at the hydroxyl and carboxylic groups of poly (vinyl alcohol) and poly (acrylic acid): Is this reaction mechanism still unclear? *J. Org. Chem.* **2009**, *74*, 3750-3757.
31. Hoch, E.; Hirth, T.; Tovar, G.E.; Borchers, K. Chemical tailoring of gelatin to adjust its chemical and physical properties for functional bioprinting. *J Mater Chem B* **2013**, *1*, 5675-5685.
32. Chen, T.; Embree, H.D.; Brown, E.M.; Taylor, M.M.; Payne, G.F. Enzyme-catalyzed gel formation of gelatin and chitosan: Potential for in situ applications. *Biomaterials* **2003**, *24*, 2831-2841.
33. Hoch, E.; Schuh, C.; Hirth, T.; Tovar, G.E.; Borchers, K. Stiff gelatin hydrogels can be photo-chemically synthesized from low viscous gelatin solutions using molecularly functionalized gelatin with a high degree of methacrylation. *J Mater Sci Mater Med* **2012**, *23*, 2607-2617.
34. Djabourov, M.; Leblond, J.; Papon, P. Gelation of aqueous gelatin solutions. I. Structural investigation. *J. Phys.* **1988**, *49*, 319-332.
35. Levett, P.A.; Melchels, F.P.; Schrobback, K.; Hutmacher, D.W.; Malda, J.; Klein, T.J. Chondrocyte redifferentiation and construct mechanical property development in single‐component photocrosslinkable hydrogels. *J Biomed Mater Res A* **2014**, *102*, 2544-2553.
36. Berneel, E.; Philips, C.; Declercq, H.; Cornelissen, R. Redifferentiation of high-throughput generated fibrochondrocyte micro-aggregates: Impact of low oxygen tension. *Cells Tissues Organs* **2016**, *202*, 369-381.
37. Sridharan, B.; Lin, S.M.; Hwu, A.T.; Laflin, A.D.; Detamore, M.S. Stem cells in aggregate form to enhance chondrogenesis in hydrogels. *PLoS One* **2015**, *10*, e0141479.
38. Zhang, J.; Yang, Z.; Li, C.; Dou, Y.; Li, Y.; Thote, T.; Wang, D.-a.; Ge, Z. Cells behave distinctly within sponges and hydrogels due to differences of internal structure. *Tissue Eng. Part A* **2013**, *19*, 2166-2175.

Chapter 5

Concluding remarks and future prospects

5.1 Concluding remarks

This study focused on the fabrication of photocrosslinkable gelatin hydrogels with different stiffness and microporous structures for cartilage tissue engineering. Firstly, the GelMA hydrogels with different stiffness were synthesized and studied for 3D culture of chondrocytes. Secondly, the highly crosslinked GelMAGMA hydrogel was fabricated with double methacrylate modified gelatin macromer to investigate the influence of crosslinking density on chondrocyte proliferation and phenotype. Finally, the microporous gelatin hydrogels were fabricated to study the effect of microporous structure on chondrocyte functions.

Chapter 1 introduces the background of conventional treatments of cartilage defect and cartilage tissue engineering. The factors and problems in cartilage tissue engineering are also reviewed. Various kinds of scaffolds, such as porous scaffolds, fabric scaffolds and hydrogels are compared. Furthermore, the advantages of photocrosslinkable gelatin hydrogel are discussed and the reasons to choose gelatin hydrogels with different stiffness and microporous structures are explained.

Chapter 2 describes the fabrication of GelMA hydrogels with different stiffness by using the macromers with different functionalization degree. In this part, the same concentration of gelatin macromer was used to prepare the gelatin hydrogels to exclude the effect of biochemical influence, such as RGD density. Chondrocytes were encapsulated in the hydrogels for 3D culture. The results of chondrocyte morphology, chondrogenic matrices secretion and chondrogenic gene expression demonstrated that gelatin hydrogels with higher stiffness were beneficial for the maintenance of chondrogenic phenotype.

Chapter 3 presents a novel method to synthesize the double methacrylate modified gelatin macromer GelMAGMA. The GelMAGMA hydrogels exhibited a high storage modulus and a slow enzymatic

degradation. Chondrocytes cultured in the GelMA and GelMAGMA hydrogels showed high cell viability. Chondrocytes in the gelatin hydrogels had round morphology and maintained cartilaginous phenotype while the secretion of ECM and chondrogenic phenotype was a little inhibited by highly crosslinked GelMAGMA hydrogel. The GelMAGMA hydrogel should be useful for strengthening hydrogel scaffolds with a low degradation rate for cartilage tissue engineering.

Chapter 4 describes the effect of microporous structures in gelatin hydrogels on chondrocyte functions. A cutting method was used to prepare gelatin hydrogel microcubes as porogen materials to introduce microporous structures in hydrogels. The combination of the microcubes and photocrosslinkable GelMAGMA hydrogel was used to prepare the microporous GelMAGMA hydrogels. The microporous hydrogels were used for 3D culture of chondrocytes. The results indicated that chondrocytes cultured in the microporous hydrogels showed higher proliferation while lower expression and production of cartilaginous matrices. Chondrocytes cultured in gelatin hydrogels without microporous structures showed high expression and production of cartilaginous matrices while low cell proliferation. Gelatin hydrogels with or without microporous structures showed different effects on chondrocytes functions.

In conclusion, photocrosslinkable gelatin hydrogels with different stiffness, crosslinking density and microporous structures were prepared for 3D culture of chondrocytes. Gelatin hydrogels could mimic the *in vivo* ECM environment and possess a good biocompatibility. GelMA hydrogels with different stiffness could be fabricated by altering the modification degree of GelMA macromers while keeping the same mass concentration. Double modified gelatin macromer (GelMAGMA) was synthesized by further binding the hydroxyl and carboxyl groups in gelatin molecules with glycidyl methacrylate to increase the photocrosslinkable double bonds in gelatin macromers. The highly crosslinked gelatin hydrogels improved mechanical properties and degradation rate of gelatin hydrogels. Furthermore, gelatin hydrogels with microporous structures were fabricated by mixing with gelatin hydrogel microcubes. The microporous gelatin hydrogels could promote the proliferation of encapsulated cells. The results of this study should provide some valuable information for the design and preparation of hydrogels for cartilage tissue engineering.

5.2 Future prospects

The study described in this dissertation was focused on the synthesis of photocrosslinkable gelatin macromers and fabrication of gelatin hydrogels with different mechanical properties or microstructures. All the conclusions obtained from this study are useful to the future design of gelatin-based hydrogels for cartilage tissue engineering and clinical applications.

In this study, the isolated bovine articular chondrocytes were used for cartilage tissue regeneration. However, it is difficult to get a sufficient number of functional chondrocytes from elderly patients with osteoarthritis. Furthermore, during 2D expansion culture, chondrocytes lose the chondrogenic phenotype. In the future work, other cell sources, such as stem cells should also be used for 3D culture in the photocrosslinkable hydrogels because stem cells can be derived from bone marrow or other tissues. After getting enough cell number by subculture, stem cells can be encapsulated in the gelatin hydrogels for 3D cultivation.

The microporous hydrogel fabricated in this study can also be used for coculture. Many literatures have reported that coculture of chondrocyte and stem cell can be used for cartilage regeneration. A small number of chondrocytes may produce some chondrogenic induction factors and promote chondrogenic differentiation of stem cells. At the same time, stem cells can secret some growth factors to promote the proliferation of chondrocytes. Therefore, the GelMAGMA hydrogels may be useful for the coculture of chondrocytes and stem cells. Because these materials and systems are biocompatible and injectable, they may also be used for tissue engineering of other tissues and organs.

List of publications

1. Xiaomeng Li, Shangwu Chen, Jingchao Li, Xinlong Wang, Jing Zhang, Naoki Kawazoe and Guoping Chen.
   3D Culture of chondrocytes in gelatin hydrogels with different stiffness.
   Polymers, 2016, 8(8): 269

2. Xiaomeng Li, Ying Chen, Naoki Kawazoe and Guoping Chen.
   Influence of microporous gelatin hydrogels on chondrocyte functions.
   Journal of Materials Chemistry B, 2017, 5, 5753-5762

3. Xiaomeng Li, Jing Zhang, Naoki Kawazoe and Guoping Chen.
   Fabrication of highly crosslinked gelatin hydrogel and its influence on chondrocyte proliferation and phenotype.
   Polymers 2017, 9(8): 309

4. Jingchao Li, Xiaomeng Li, Jing Zhang, Naoki Kawazoe, Guoping Chen.
   Induction of Chondrogenic Differentiation of Human Mesenchymal Stem Cells by Biomimetic Gold Nanoparticles with Tunable RGD Density.
   Advanced Healthcare Materials 2017, 1700317

5. Jianmin Yang, Jingchao Li, Xiaomeng Li, Xinlong Wang, Yingjun Yang, Naoki Kawazoe, Guoping Chen.
   Nanoencapsulation of individual mammalian cells with cytoprotective polymer shell.
   Biomaterials 133, 253-262

6. Jianmin Yang, Jingchao Li, Xinlong Wang, Xiaomeng Li, Naoki Kawazoe, Guoping Chen.
   Single mammalian cell encapsulation by in situ polymerization
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