Abstract

Extracellular-matrix Cryogels for Cartilage Tissue Engineering

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In this thesis, we investigated highly porous extracellular matrix (ECM) cryogels for cartilage tissue engineering. For ECM cryogel fabrication, methacrylated chondroitin sulfate (MeCS) or methacrylated hyaluronic acid (MeHA) was co-polymerized with PEG-based or gelatin-based system via free radial initiation under freezing condition. This procedure induced ice crystal formation resulting in macroporous structures within ECM-cryogels. The developed PEG-based ECM-cryogel system exhibited average macroporosity of 75% and supported the chondrocyte infiltration. Additionally, gelatin-based ECM cryogel showed average porosity of 65%. When rabbit chondrocytes were cultured on PEG-based ECM cryogels, PEG/CS cryogels stimulated *aggrecan* gene expression and GAG accumulation, whereas PEG/HA cryogels stimulated *type II collagen* gene expression and collagen accumulation. Presence of CS in gelatin-based cryogels showed strong upregulation in all types of cartilage related genes (*type II collagen, type X collagen, aggrecan, HAS2, PRG4, Link protein*). Gelatin/HA cryogel showed significant increase in *HAS2, link protein* and

type II collagen. These results demonstrated that the ECM cryogels can be utilized to promote specific ECM secretion for cartilage tissue engineering.

Keywords: Cryogel, Hyaluronic acid, Chondroitin sulfate, Cartilage Tissue engineering, Poly (ethylene glycol) diacrylate, Gelatin

***Student Number*: 2014-20595**

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CHAPTER ONE: THE SCIENTIFIC BACKGROUND AND RESEARCH PROGRESS

1.1 Cartilage tissue engineering

Cartilage is an avascular structured tissue with limited self-regeneration capacity, which makes it a key target for cell-based therapy and tissue engineering[1] A current articular cartilage repair strategy is comprised of autologous chondrocytes transplantation into a lesion site followed by periosteal flap covering [2]. In addition, scaffold-based treatment options are becoming more accessible for patients with focal cartilage defects[3]. However, these clinical options often result in additional surgical interventions due to inadequate long-term stabilities of implanted cells or biomaterials [4]. Consequently, a strategy based on the biomimetic design of scaffolds for optimal cellular microenvironments is on demand in order to preserve the chondrocyte phenotypic stability and to promote cartilage regeneration.

1.2 Extracellular matrix (ECM) components for scaffold

In biology, extracellular matrix (ECM) is non-cellular environment and provides structural support for tissue. In addition to providing physical support for cells, ECMs actively participate in regulation of cell functions such as proliferation and homeostasis. To understand the function of ECM, the understanding of ECM's components was required. Cartilage ECM is composed of mainly two components. First, the collagenous network is responsible for tensile strength of cartilage matrix. A typical collagen molecule is elongated with a triple helical structure, which plays a dominant role in maintaining the biological and structural integrity of cartilage tissue [5, 6].

Second, proteoglycans are responsible for osmotic swelling and elastic properties in cartilage tissue [5]. Proteoglycans are macromolecules composed of a central core protein with carbohydrate chains, called glycosaminoglycans (GAGs), covalently attached. Generally, the ECM proteoglycans are divided into two major groups: aggregating and non-aggregating groups. In cartilage, the aggregating proteoglycan, aggrecan, is highly expressed. Also, Chondroitin sulfate and hyaluronic acid are major components of GAGs in cartilage tissue. In order to create an ideal microenvironment for cartilage tissue engineering, tissue-like compliance along with interconnected macroporous structures to allow 3D cellular growth would be desirable [7]. To serve as a biological substrate, scaffold should promote ECM deposition, allow sufficient oxygenation, facilitate nutrient exchanges, and provide cellular homeostasis [8, 9]. The choice of material may play a key factor in cell scaffold design. Recently, biomaterials for cartilage tissue engineering have focused on the development of macroporous cryogels. Macroporous structures within cryogels can be formed as a result of cryogenic treatment during a polymerization process [10]. The macroporosity allows influx of high-molecular-weight solutes and nutrients, as well as transport of cellular waste. This macroporosity also enhances the extracellular matrix (ECM) deposition from cells [11]. Furthermore, these structural advantages allow a considerable degree of mechanical flexibilities of hydrogels by altering pore sizes and swelling ratios [12]. Up to date, varieties of synthetic, natural, or biosynthetic biomaterials such as gelatin [13], poly (ethylene glycol) diacrylate (PEGDA) [14], chitosan [10], and agarose [15] have been utilized as starting biomaterials for cryogel fabrications. In particular, HA is a high molecular naturally-occurring polysaccharide, which exerts biological functions such as

maintaining elasto-viscosity of liquid connective tissue, controlling tissue hydration and water transport, and assembling proteoglycans in ECM [16, 17]. Also, HA incorporated hydrogels have been shown to enhance bioactive properties by binding through cell surface receptors such as CD44 [18] and HA incorporated PLGA scaffold showed three times greater bioactivities than normal PLGA scaffolds [19]. Chondroitin sulfate (CS) is sulfated glycosaminoglycan (GAG) that is normally found in articular cartilage and contributes to tensile strength of cartilage with unique extracellular matrix molecules [20]. The effect of chondroitin sulfate in patients with osteoarthritis is stimulation in the synthesis of proteoglycans and decrease in catabolic activity of chondrocytes by inhibiting proteolytic enzymes and other factors that contribute to cartilage matrix damage [21]. CS incorporated hydrogel supported these positive effect. The PVA hydrogel containing CS showed improved cell viability compared to PVA hydrogel [22] and CS with PEG-RGD hydrogel showed increasing trend of cellular proliferation for 3weeks and produced highest level of GAG accumulation [23].

Hence, we fabricated cryogels based on extracellular matrix components which have been frequently used for cartilage tissue engineering due to their key biological advantages.

1.3 Cryogels

Cryogels have interconnected macropores and their fabrication method is initiated from aqueous solution in freezing state at subzero temperature by crystallizing of a solvent with monomers. The formation of cryogels follows these steps [12]. First, the reaction mixture containing gel-forming agents is frozen at subzero temperature. Secondly, the crystals of frozen solvent perform as pore-forming agents and initiate cryo-

polymerization untill they make contact with surface of other crystals. After polymerization is saturated, the ice is allowed to finally thaw and supermacroporous cryogel is fabricated. Cryogels have recently been used as cell-scaffold due to their macroporous structure, allowing the effective mass transport of macromolecular solutes [24] and the migration of cells [25] by providing sufficient surface area, high biocompatibility [26] and good mechanical properties [15] owing to its strong association with water [27].

It is attractive to apply cryogels to tissue engineering application because it satisfies all critical parameters for cell-scaffold. First, studies on cartilage tissue engineering have demonstrated the potential future application of cryogel for repairing defects. For instance, multilayer of gelatin-chitosan hydrogel induced formation of new cartilage in subchondral bone defect for within months and regenerated tissue showed similarity to native cartilage-bone tissue [28]. In addition, chitosan-agarose-gelatin cryogel scaffold has shown good potential for treatment of subchondral defect. The cell-laden cryogels successfully repaired subchondral cartilage defects and had good integration between subchondral bone and adjacent native cartilage [29]. Secondly, cryogels are also frequently used for bone tissue engineering. For instance, gelatin and hydroxyapatite (osteoconductive component of bone) based cryogel was subcutaneously implanted in rat model to regenerate bone [30]. Additionally, gelatin-hydroxyapatite cryogels with BMP2 was applied to craniofacial bone defects [31]. In another study, PTAC (polyvinyl alcohol-tetraethtylorthosilicate-alginate-calcium oxide) incorporated biocomposite cryogel not only enhanced bone regeneration at the defect site, but it was also simultaneously integrated with pre-existing tissues within 4weeks [32]. Based on

these findings, in the field of tissue engineering, the selection of materials has great effect on cellular responses due to their favorable characteristics.

1.4 Aims of research

In this work, we evaluate the potential of multiple components of cryogels based primarily on synthetic polymer, Poly (ethylene glycol) diacrylate (PEGDA), and natural polymer, gelatin methacrylate (GelMA). The introduction of PEGDA in the formation of cryogels is to increase the mechanical properties. In addition, as incorporated with HA or CS, double-network hydrogel produced extremely high mechanical strength [33]. The mechanical tunability of cryogel may give a chance to be used as tissue engineering applications. Gelatin is preferably selected due to several reasons. It is natural biodegradable polymer derived from collagen and contains RGD binding sequences, allowing cells to bind directly to the GelMA cryogels [34, 35]. The present study tests the hypothesis that the incorporation of HA and CS may enhance cell response for better cartilage specific molecule deposition or gene expression. With ECM biomolecules (CS, HA), we demonstrated cell-laden cryogels generated such suitable cell responses, like cartilage specific gene expression and inducing cartilaginous ECM molecules for application in tissue engineering through chapter 2&3. Finally, in future investigations, applying these cryogels on the rabbit osteochondral defect model will hopefully produce better cartilage tissue regeneration and enhanced integration among tissues.

CHAPTER TWO: Poly (ethylene glycol) diacrylate based cryogel for cartilage tissue engineering

2.1 Introduction

To create an ECM-biomimetic environment within cryogel structure, HA and CS were functionalized with metharylate group and exposed under radical polymerizing condition with PEGDA at subzero temperature. The presence of ammonium persulfate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED) catalyzed the polymerization during ECM-cryogel formation. Synthetic polymer, PEGDA have been widely used as cell scaffold due to their high permeability to oxygen, nutrients, making them particularly attractive as scaffolds in tissue engineering applications [36]. We demonstrated the–chondrocytes seeded in PEG based cryogels showing such a suitable cell response and inducing cartilaginous ECM molecules for application in tissue engineering.

2.2 Materials and methods

2.2.1 Isolation and culture of rabbit chondrocytes

Rabbit chondrocytes were harvested from articular cartilage biopsies of New Zealand white rabbits (Koatech Laboratory Animal Company, Korea). Cartilage fragments were dissected and digested with 0.2% (w/v) collagenase type II (Worthington, Lakewood, NJ) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL) for 16 hours. Isolated chondrocytes were seeded on tissue culture plates at 5×10^5 cells /ml at 37°C in a humidified 5% (v/v) CO_2 incubator, and the cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 1% penicillin/streptomycin (Gibco-BRL), 1% HEPES (Gibco-BRL), 1% MEM Non-Essential Amino Acid (MEM NEAA; Gibco-BRL), 0.2% l-proline (Sigma-Aldrich, Saint Louis, MO) and 0.2% l-ascorbic acid (Sigma-Aldrich). The medium was replaced every 2 days. Passage 1 (P1) cells were used for this study.

2.2.2 Methacrylation of chondroitin sulfate and hyaluronic acid

Methacrylated hyaluronic acid (MeHA) and methacrylated chondroitin sulfate (MeCS) were synthesized as previously described[23]. In brief, HA (Mw 64,000; Life Core Technologies) was mixed at 1% (w/v) with phosphate-buffered saline (PBS; Gibco-BRL) and stirred until being completely dissolved, followed by addition of glycidyl methacrylate 2% (v/v) (GMA; Sigma-Aldrich) for methacrylate group incorporation. The reaction solution was stirred vigorously for 8 days, then the resulting clear solution was dialyzed against water with 1000MW dialysis membrane for 2 days with several changes of water during dialysis. The final solution was frozen at -80°C and lyophilized to obtain MeHA. For MeCS, the same protocol of MeHA synthesis was applied. CS (Mw 20,000~40,000; Tokyo Chemical Industry) was mixed at 10% (w/v) with PBS and

GMA 10% (v/v) was added. Then the mixture was reacted for 11 days, while remainder steps were the same.

2.2.3 Fabrication of PEGDA based cryogels

ECM-based cryogels were prepared by mixing 1:1 volume ratio of PEGDA 20% (w/v) with either MeHA 1% (w/v) or MeCS 10% (w/v). To these mixed solutions at 4°C, stock ammonium per-sulfate 10% (w/v) (APS; Sigma-Aldrich) solution and N,N,N',N' -tetramethylethylenediamine (TEMED; Sigma-Aldrich) solution were added to the final desired concentrations of 4% (v/v) , 0.2% (v/v) respectively. To start cryopolymerization, this polymer solution was put into cylindrical polyethylene mold and placed in the freezer (-20°C) for 20h. Interconnected and macroporous cryogels (diameter=8mm, height=4mm, and total volume = 200 µl) were synthesized by thawing. Prior to seeding the cells, cryogels were sterilized with UV for 20 min and washed several times with PBS to remove unreacted residues. Cells were seeded onto three different types of cryogels: PEGDA (10% w/v), PEGDA + MeHA (P-MeHA), and PEGDA + MeCS (P-MeCS) at a concentration of 1×10^6 cells /construct in a drop wise manner[14]. The samples were cultured in 1.5ml of chondrocyte medium (DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% HEPES, 1% MEM NEAA, 0.2% l-proline and 0.2% l-ascorbic acid) at 37°C in 5% CO_2 environment, and the medium was changed every 2 days for up to 3 weeks *in vitro*.

2.2.4 Rheological and mechanical analysis

Rheological measurement was performed using advanced rheometric expansion system (TA Instrument, ARES). The measurement was performed in parallel plate geometry. The time sweep test had been performed from 0 to 300s. To evaluate gelation point with a rheometer, specimens were prepared in the solution state (PEGDA 800µl, APS 40 µl, TEMED 2µl respectively) and placed between the rotating plate (diameter 25mm). Then, the upper stationary plate was lowered to a measurement gap of 1.595mm at room temperature and the shear rate was kept 10[1/s].

The mechanical properties of cryogels in the wet state were determined using Instron (5900S, USA). Compressive modulus was calculated from the linear region in stress–strain curve using a theoretical model.

2.2.5 Swelling ratio analysis

For swelling studies, cryogels were swollen in PBS for overnight, and wet weight was measured after removing the surface water with weighing papers. Then, dry weight of freeze-dried hydrogels was measured. The swelling ratio was calculated by the following equation.

$$\text{Swelling ratios (Q)} = \frac{wet\ weight\ of\ equalibrated\ cryogel\ in\ PBS}{weight\ of\ the\ dried\ cryogel}$$

2.2.6 Scanning electron microscopy analysis

For SEM analysis (JSM-6701F; JEOL), cryogels were lyophilized before gold coating and adhered on the sample stubs using carbon tapes. Cell-laden cryogels (cell density;

5×10^5cells/construct) dehydrated with hexamethyldisilazane (HMDS) to preserve the cellular morphology as previously reported[37]. The pore size and porosity were determined using scanning electron micrographs associated with Image J software and MATLAB.

2.2.7 Live/dead assay

Live/Dead Cell viability/Cytotoxicity kit (Molecular Probes; L3224) was used following the manufacturer's protocol. Live cells were stained by green fluorescent calcein AM and red fluorescent Ethidium homodimer-1 (Ethd-1) stained dead cells. To determine the degree of cell viability, cell-laden cryogel (8×10^5cells/construct) was measured several times with Image J software.

2.2.8 Biochemical assay

Biochemical analysis was performed on cell-laden cryogels to analyze the amount of DNA, GAGs (glycosaminoglycans), and collagen. Samples were collected after 3weeks and lyophilized for 24h and then digested with 1ml of papain solution (1ml/construct; 125µg/ml; Worthington Biomedical) as physically crushed for 16h at 60°C. DNA content was quantified using a Quanti-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™). The GAG content was also quantified using dimethylmethylene blue (DMMB) spectrophotometric assay at A525, as previously described[38]. To detect newly secreted GAG from P-MeCS cell laden cryogels, we prepared acellular samples

(PEGDA+MeCS) to subtract GAG content from total GAG content. Total collagen content was determined by measuring the amount of hydroxyproline within the constructs after acid hydrolysis and reaction with p-dimethylaminobenzaldehyde and chloramine-T, as previously described [39]. Accumulation of DNA, GAG, and collagen contents were normalized to the dry weight of each sample.

2.2.9 Real-time pcr analysis

Gene expression of *Aggrecan*, *Hyaluronic acid synthase 2, Type II collagen*, *Lubricin* and *Link protein* were analyzed after 3 weeks of culture (n=3). Total RNA was extracted from each hydrogel with Trizol and reverse transcribed into cDNA using M-MLV cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. Using SYBR Green PCR Mastermix and ABI StepOnePlus^{TM} Real time PCR system, cDNA was amplified by rabbit specific primer for *Aggrecan*, *Hyaluronic acid synthase 2*, *Type II collagen*, *Lubricin* and *Link protein* genes. *GAPDH* gene was used as a control and gene expression level was calculated as $-2^{\Delta\Delta Ct}$. The rabbit specific primers are listed in Table 1.

2.2.10 In vivo transplantation

For *in vivo* analysis, female BALB/c-nude mice at 4 weeks of age (16-20g body weight, OrientBio, Gyeonggi province, Republic of Korea) were anesthetized and cryogel samples (Cell density: 2×10^6cells/construct) were implanted subcutaneously.

2.2.11 Histological analysis

Cell-laden cryogels were fixed overnight with 4% paraformaldehyde, dehydrated with a serial concentration of 50%, 75%, 90%, 95%, 100% ethanol, and embedded in paraffin. *In vitro* samples were cut into 10μm sections and stained with hematoxylin and eosin (H&E) or Safranin-O. For *in vivo* tissues, constructs were fixed in 4% paraformaldehyde for 24h and placed in 20% (w/v) sucrose (Sigma-Aldrich) at 4°C overnight. Then, samples were treated with an infiltration solution (1:1 volume ratio of O.C.T compound; CellPath, Powys, UK and 20% (w/v) sucrose) at room temperature overnight. After transferring samples to cryomold, more OCT was carefully dropped onto the specimen until completely covering. Tissue samples were then cryosectioned into 10μm thick sections and stained with H&E and Safranin-O solutions.

2.2.12 Immunohistochemical staining analysis with aggrecan and collagen type II

For immunofluorescence staining, sections were blocked with blocking solution containing 1% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100, and 10% (v/v) normal goat serum for 45 min. Sections were incubated with monoclonal anti-aggrecan antibody (1:500 dilution; Novus Biological, Littleton, USA, NB600-504) or monoclonal anti-collagen type II antibody (1:500 dilution; Novus Biological, Littleton,

USA, NBP2-33343) at 4°C for overnight. Sections were then incubated with goat anti-mouse Alexa Fluor 488 (1:500 dilution; Life Core Technologies, 1563682). The nuclei were stained with DAPI (Sigma-Aldrich), and images were obtained using LSM 780 confocal micro-scope (Zeiss).

2.2.13 Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA single factor) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

2.3 Results & discussion

2.3.1 Preparation and characterization of cryogels

ECM-based biomaterials have attracted a great deal of attention in the field of regenerative engineering. A significant progress has been made in designing, synthesizing, and using these materials for cartilage tissue engineering applications [23, 40]. ECM-based hydrogels have shown significant potential towards tissue engineering applications. Hyaluronic acid (HA) and chondroitin sulfate (CS) are essential components of the cartilage ECM. In this study, ECM-based cryogels were fabricated through radical polymerization in addition to a phase transition undergoing subzero temperature from freezing. For methacrylation of HA and CS, GMA (10% v/v) with reactive hydroxyl group was mixed with HA (1% w/v) or CS (10% w/v). H^1-

NMR spectra of MeCS confirmed presence of acrylate group peaks at 6.140 ppm and 5.711 ppm, and H^1-NMR spectra of MeHA group showed acrylate peaks at 6.154 ppm and 5.719 ppm (Figure 1.1). MeHA (1% w/v) was mixed with PEGDA (20% w/v) in 1:1 ratio to form HA-based cryogel (P-MeHA). Likewise, MeCS (10% w/v) was mixed with PEGDA (20% w/v) in 1:1 ratio to form CS-based cryogel (P-MeCs). Crygels were formed via ammonium persulfate (APS) and N,N,N',N' - tetramethylethylenediamine (TEMED) mediated polymerization in freezing condition at -20°C. For control hydrogel, PEGDA cryogel was utilized with 10% w/v. During the cyrogel formation, the ice crystals act as porogens, which ultimately contribute to the interconnected porous structure (Figure 2.1). PEGDA cryogels, P-MEHA cryogels, and P-MeCS cryogels were lyophilized prior to cell seeding. In swelling condition, all the cryogels significantly increased their swelled volume and were opaque (Figure 2.2). Overall properties of cryogels were dependent on the balance between the polymerization rate and the freezing rate. During the sol-gel transition, solution state lead to formation of three dimensional gel scaffold. It is important to check an optimal gelation point to maintain cross-linking density, because it may affect the structure regulating the mechanical properties. In the case of fast cross-linking condition, it has resulted in brittle structures, low porosity, and inferior mechanical strength [41]. In order to assess the polymerization time, the rheological properties of polymeric solutions underdoing polymerization were monitored with constant shear rates at 10 [1/s]. Once the polymerization was finished, the viscosity of cross-linked cryogels have shown a consistent value. At an incomplete gelation point, the viscosity continues to rise abruptly with increasing cross-link density until the reaction completes. Complete

polymerization time was measured by a shift in phase transition, as it turned from the sol to gel state. When polymer solutions were mixed with an initiator, the viscosities of PEGDA, P-MeHA, and P-MeCS clearly increased from 0 to 10^0 Pa-s (Figure 2.3). Consequently, we demonstrated that the cross-linking reaction stopped at similar reaction time around 200s in all groups (Figure 1C). After 200 seconds of polymerization time, viscosities remained constant indicating a completed gelation (Figure 1C). Additionally, we examined the cryogel properties by measuring the equilibrium swelling ratios, Q, by immersing them in PBS overnight. The swelling behavior of cryogels is determined by several physicochemical factors and can demonstrates its porous interior characteristics, which has influence on the viability of seeded cells [42]. In our study, we adjusted the swelling ratios of cryogels so that the ratios are similar to one another. In this way, each gel's original water retention capacity does not affect it's ability in holding cells. PEGDA, P-MeHA, and P-MeCS cryogels showed q of around 10 (Figure 1D). Interestingly, compressive moduli of acellular cryogels were significantly increased by the addition of MeHA or MeCS in to the PEGDA (Figure 1E). The Young's modulus of PEGDA cryogel, $66.50 \pm \mathbf{0.27}$ kPa, was considerably smaller than those of P-MeHA and P-MeCS cryogels. Even with small amounts of MeHA and MeCS, each cryogel showed significant difference in compressive modulus when compared with PEGDA cryogels. This phenomenon may be due to multi-functional branches of HA and CS that can make interpenetrating polymer network (IPN). IPNs are cross-linked polymers comprising two or more networks partially interlaced. One of the main advantages of IPN structure is providing dense hydrogel matrices that have stiffer and tougher mechanical properties [43]. The greatest enhancement in the elastic

modulus was seen in P-MeCS cryogels. These results can be explained by higher concentration of methacrylated branches in MeCS, resulting in increased water retention by negatively charged CS. These data clearly indicated that the stiffness of cryogels is changeable across a full range of Young's modulus from 66.54 ± 0.27to 177.07 ± 17.14kPa by varying the ECM mimetic polymer components. We further characterized the structures of PEGDA, P-MeHA, and P-MeCS cryogels by SEM (Figure 2A-F). Through a simple cryo-polymerization at subzero state, we were able to fabricate interconnected macroporous cryogels that contained high porosities. By manipulating the temperature or concentration of chemical initiators, we were also able to control poresizes. The internal structure of croygels showed similar morphology in all types of cryogels. This may be due to a rapid homogeneous-mixing in the polymer solution with an identical gelation point. The pore size of each scaffold has great effects on the cell proliferation, cell metabolism, amount of GAG secretion and the increase of cartilage related gene expression level. According to the previous studies, cell scaffolds with larger pore sizes served as effective tools for supporting the chondrocyte phenotype and retaining production of ECM molecules[44]. Not only the pore size, but also a higher pore volume fraction (1-porosity) resulted in increasing cell proliferation due to the effect of higher surface area/unit volume ratio [45]. From SEM micrographs, we observed a wide range of the pore sizes and heterogeneous structure, on each group because the micro pores that are formed among the polymer chains. In an aqueous medium, the fluid easily diffuses into cryogels due to its interconnected pores. When compared to P-MeHA and P-MeCS, PEGDA, cryogels showed porous interior (up to 71.94% porosity) with larger pore diameters ranging as 89.90±30.57μm. P-MeHA

and P-MeCS cryogels had smaller pore diameters compared to PEGDA cryogels. Further, we evaluated exact pore sizes by Image J software program. P-MeHA had an average diameter of 58.68±17.57 μm and P-MeCS had an average diameter of 62.87±18.75 μm. Furthermore, P-MeHA and P-MeCS resulted in similar porosity measurement (Figure 2G).

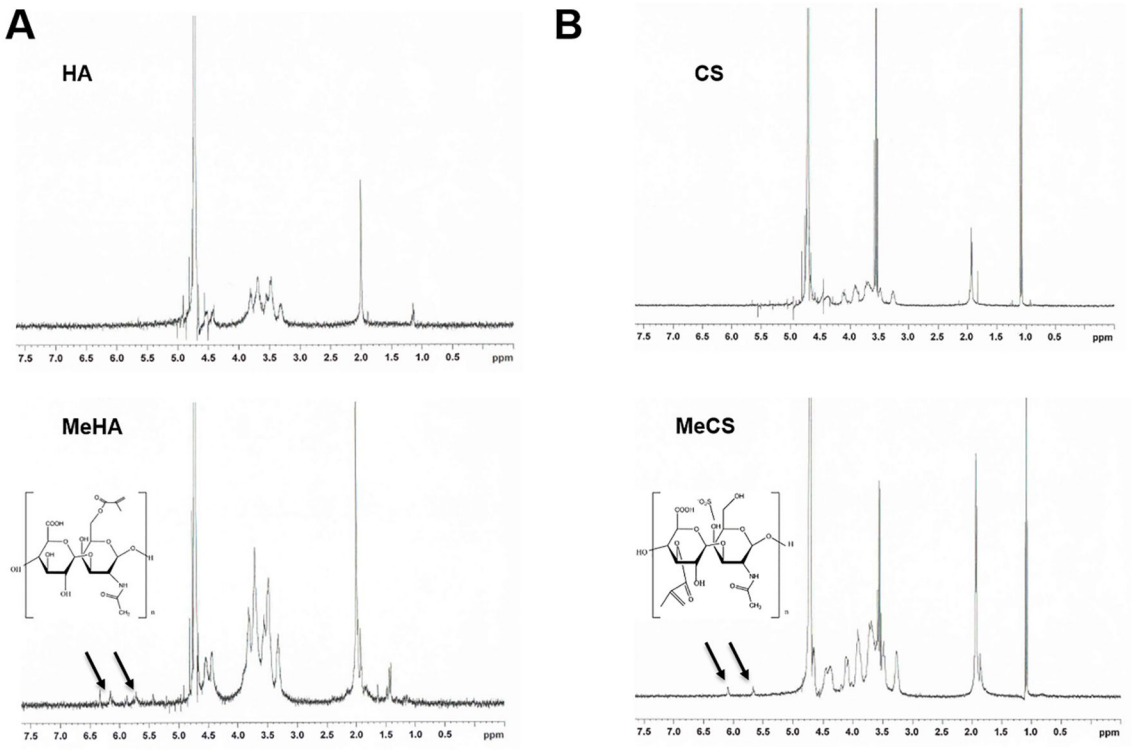


Figure 1.1 ^{1}H-NMR spectra image of CS/ MeCS and HA/MeHA.

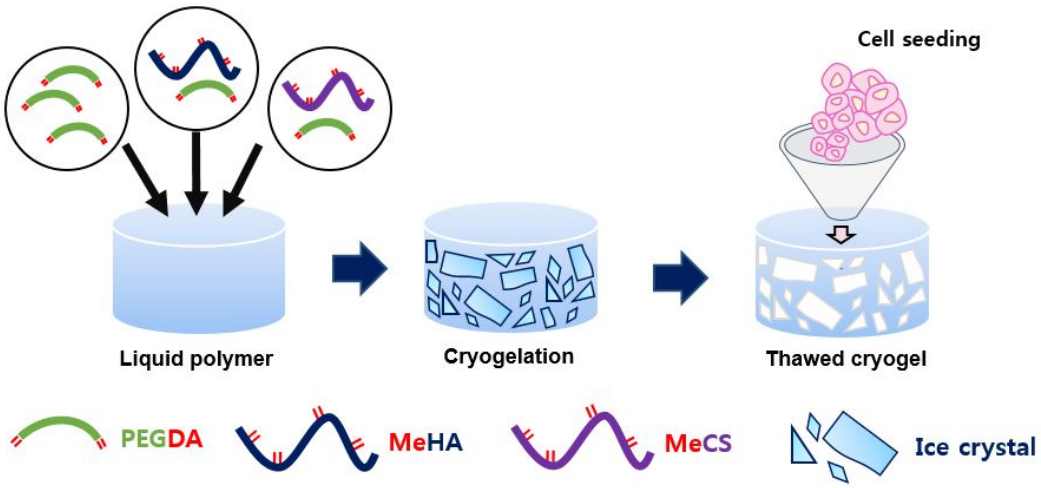
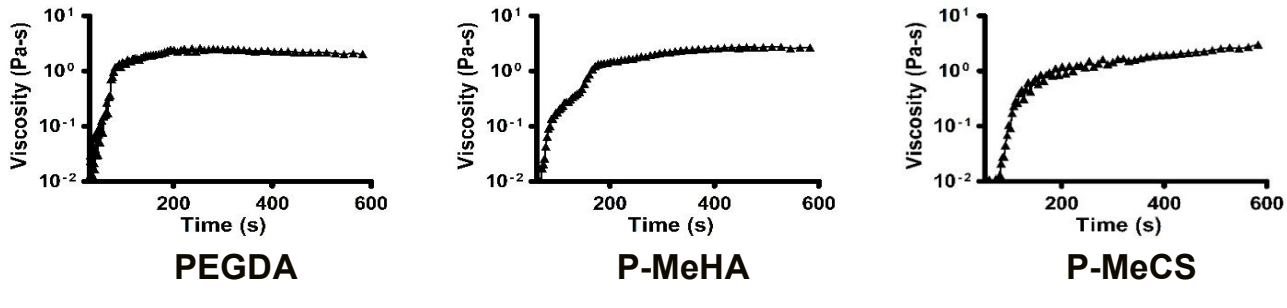


Figure 2.1 Schematic of interconnected macroporous cryogelation process:

polyethylene glycol, chondroitin sulfate and hyaluronic acid were modified with acrylate groups to allow a radical polymerization along with the presence of radical initiators (APS and TEMED) in the frozen state. PEGDA, P-MeHA and P-MeCS were mixed with APS/TEMED and formed ice crystals at -20°C for 20hours.

Figure 2.2 Images of swollen (right) and lyophilized (left) cryogels. (PEGDA, P-MeHA and P-MeCS respectively)

Figure 2.3 Viscosity of PEGDA, P-MeHA and P-MeCS swollen cryogels with time



dependence.

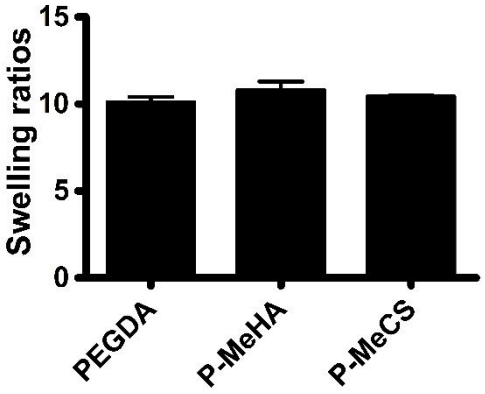


Figure 2.4 Swelling ratios of three types of swollen cryogels in PBS for 24hrs. A similar swelling ratio was observed among the cryogels. Error bars represent the standard deviation on the mean for n=4.

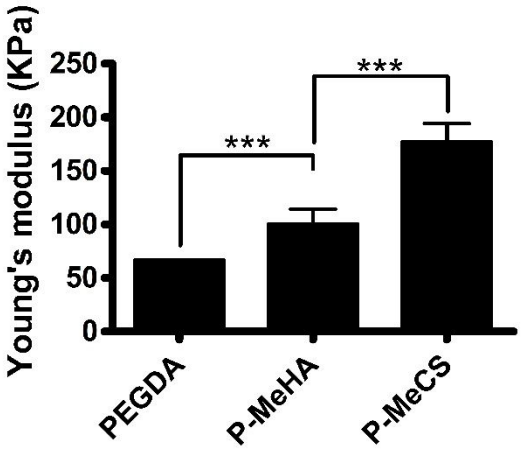


Figure 2.5 Young's modulus of PEGDA, P-MeHA and P-MeCS cyogels without cells. Error bars represent the standard deviation on the mean for n=4. *** $p < 0.005$

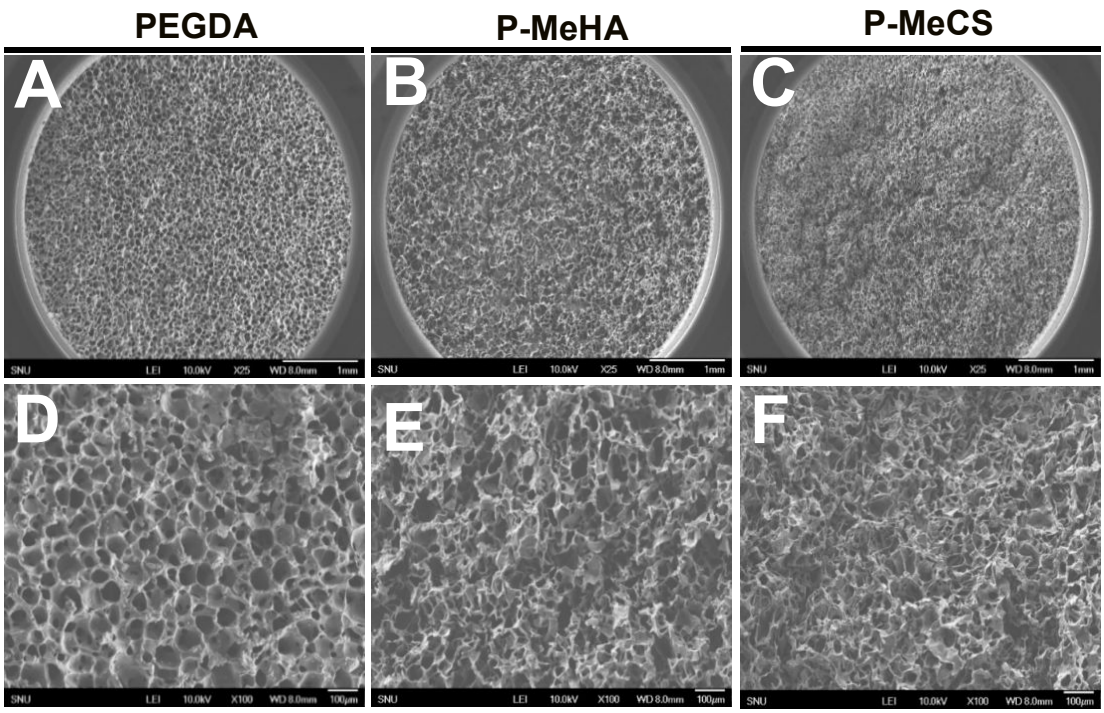


Figure 3.1 A scanned electron microscopy image of internal morphology of ECM-cryogel with low resolution **(A-C,** scale bar =1000µm**)** and high resolution **(D-F,** scale bar =10µm**)**.

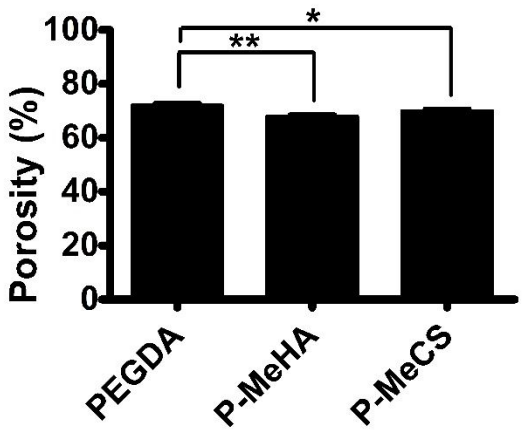


Figure 3.2 The porosity (%) of each sample was calculated using MATLAB. Error bars represent the standard deviation on the mean for n=3. * $p < 0.05$, ** $p < 0.01$

2.3.2 *In vitro* cell viability on cryogels

As a next step, the viability and seeding efficiency of the rabbit chondrocytes on ECM-based cryogels were examined. Lyophilized cryogels were immersed in chondrocyte containing medium (1 million cells/construct) and allowed to swell for 3 hours. Chondrocytes were then allowed to adhere onto the cryogels for 24 hours. Viability and cell distribution within the cryogel structure were also examined (Figure 4.1). Cells were distributed evenly within the cryogel structures indicating that the macroporous structures of cryogels allowed even infiltration of chondrocytes. SEM analysis of adherent cells within each cryogels showed that cells maintained round morphology, which is favorable for maintaining the chondrocyte phenotype. In addition, Live/dead assay indicated the viabilities over 95% in all cryogels conditions (Figure 4.2).

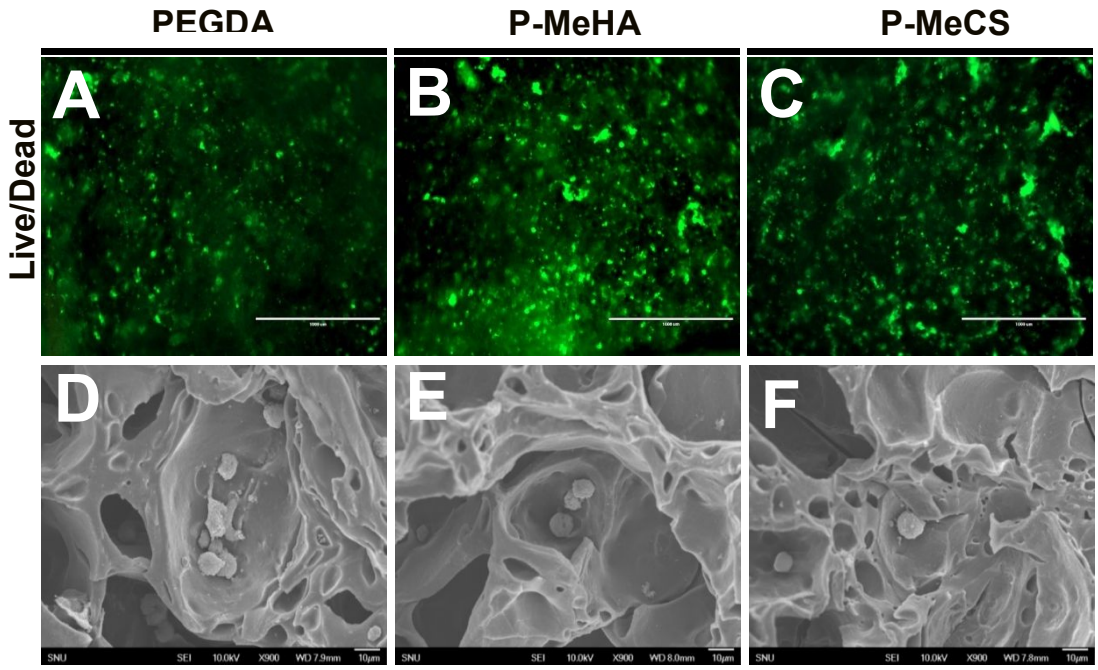


Figure 4.1 Analysis of cell viability and cell adhesions on ECM-cryogels. **(A-C)** In live/dead images (scale bar =1mm), living cells represent green; dead cells represent red. **(D-F)** Images of cells adhered well over the cryogel construct after 24 hours.

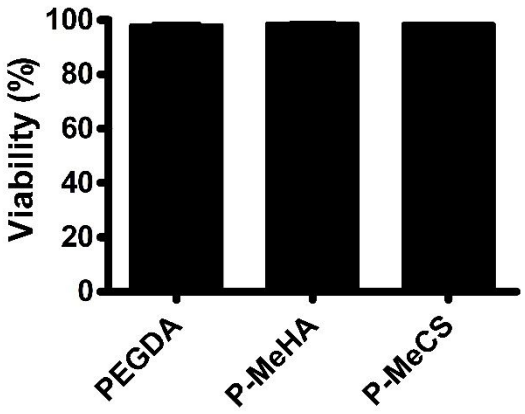
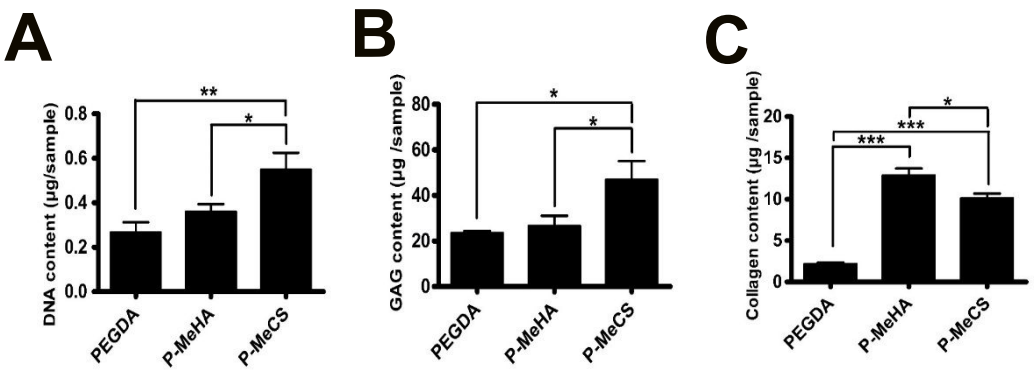


Figure 4.2 Cell viability was calculated as (alive cells/ total cells) × 100%.

2.3.3 ECM-specified cartilage matrix synthesis

Next, we further evaluated the in vitro chondrocyte responses in ECM-based cryogels. First, the proliferation of seeded cells was examined by DNA content using Pico Green

assay for 3weeks. Both P-MeHA and P-MeCS served as a suitable support system for



chondrocytes in depositing ECM matrix. There was an increasing trend of cell proliferations by MeHA or MeCS incorporation. P-MeCS cryogels resulted in the highest amount of DNAs (Figure 5.1.A). Similar to the DNA measurement, both MeHA and MeCs increased the GAG accumulation. P-MeCS had the highest amount of GAG accumulation (Figure 5.1.B). In addition, we measured the accumulated collagen by examining hydroxyproline content. Only a small amount of collagen was detected in PEGDA cryogels. Interestingly, P-MeHA resulted in the highest collagen content of 12.88 µg per construct (Figure 5.1.C). P-MeHA cryogels induced the highest production of collagen content, even though DNA content was not the highest. MeCS and MeHA were prominently appeared to have positive effects on the synthesis of GAG and collagen. Especially, the addition of HA into cryogels enhanced the cell metabolism with much enriched GAG [46]. Among all biochemical assays, PEGDA cryogels showed the lowest value due to performing as structural stability with no ability to accumulate cartilage specific proteins such as GAG and collagen. The exact values for GAG and collagen contents were also normalized to dry weight of the samples.

Figure 5.1 Biochemical analysis of the cryogel after 3weeks of *in vitro* cultivation. **(A)** DNA content, **(B)** GAG content and **(C)** Collagen content was normalized to the dry weight of cryogels. Error bars represent the standard deviation on the mean for n=3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

2.3.4 ECM-specified cartilage gene expressions

In order to confirm the biochemical observation, we evaluated the gene expression level of chondrocytes in each cryogel system. Cartilage related genes such as aggrecan, HAS2, type II collagen, lubricin and link protein were examined. Each cryogel cultured up to 3 weeks and gene expression was normalized to each cryogel in one week of culturing (Figure 6.1.A-E). The results showed that the expression level of cartilage – related genes were highly dependent on the cryogel composition. Expression of aggrecan, HAS2, type II collagen, lubricin and link protein genes were significantly upregulated in cryogels containing MeHA in D21 compared to that in D7. More specifically, within type II collagen primarily found in cartilages, the expression was 5 fold higher in P-MeHA consturction compared to PEGDA and P-MeCS showing an upregulated trend (Figure 6.1.C). Aggrecan was highly upregulated (10-fold increase) by the addition of MeCS compared to PEGDA and P-MeHA (Figure 5A). The expression in PEGDA cryogel showed similar trends as both type II collagen and aggrecan. Lubricin, also known as proteoglycan4, plays a significant role in joint lubrication. Expression of lubricin was higher in cryogel containing MeHA at D21 compared to D7 sample (Figure 6.1.D). HAS2 handles the polymerization and deposition of hyaluronic acid in the extracellular matrix. The presence of MeHA in

cryogels resulted in significantly higher HAS2 gene expression, while the other groups showed a decrease (Figure 6.1.B). A cartilage link protein, also known as hyaluronan or proteoglycan link protein1, functions as a stabilizer for the interaction between aggrecan and hyaluronic acid [47, 48]. A construct with MeHA and MeCS expressed a higher level of link protein than those without (Figure 6.1.E) them. Especially, a strong expression of link protein was observed in P-MeHA cryogels. Lastly, there is no significant difference between D7 and D21 in PEGDA cryogel except lubricin gene. These results indicated that the incorporation of MeHA and MeCS, ECM- based cryogels were highly dependent on the cryogel composition. Generally, normal hyaline articular chondrocytes have been shown to possess the predominant amount of aggrecan and type II collagen [49]. We observed that both P-MeHA and P-MeCS were upregulated higher than PEGDA cryogels between aggrecan and type II collagen gene expression in native cartilage tissue because of modified microenvironment. According to previous literatures, HAS2 appears to be one of the most important synthases because they synthesize such a high molecular weight of HA which accounts for a major portion of proteoglycan [50]. We observed that HAS2 was expressed at the highest level in cryogels containing MeHA while other groups were down-regulated. Next, it has been demonstrated that the link protein greatly strengthened the binding of proteoglycans to hyaluronate chain resulting in the formation of proteoglycan aggregation [51]. With these functions, we observed that P-MeHA cryogel resulted in increased expression level of link protein. The complex of all these three, – HA, aggrecan, and link protein— is important in normal cartilage because those three components undergo turnovering to healthy cartilage [51]. By other studies, gelatin with methacrylated HA hydrogel

showed a higher expression level than methacrylated CS in lubricin [52]. Similarly, P-MeHA cryogels showed a higher gene expression level in lubricin, which is crucial as enhancing low-friction properties in cartilage [53]. This result means that MeHA regulates the cell metabolism to promote cartilaginous characteristics. For MeCS construct, chondroitin sulfate—being superior to type C and having a synergistic effect with inherent materials' property—was used, resulting an markedly increased mRNA expression of type II collagen as P-MeCS cryogel showed. Further, P-MeCS cryogel showed up-regulation of aggrecan—one of the major structural components of cartilage [20, 53]. From these results, we confirmed that hyaluronic acid and chondroitin sulfate, in the presence of chondrocytes, have a different effect on cartilage specific gene expressions.

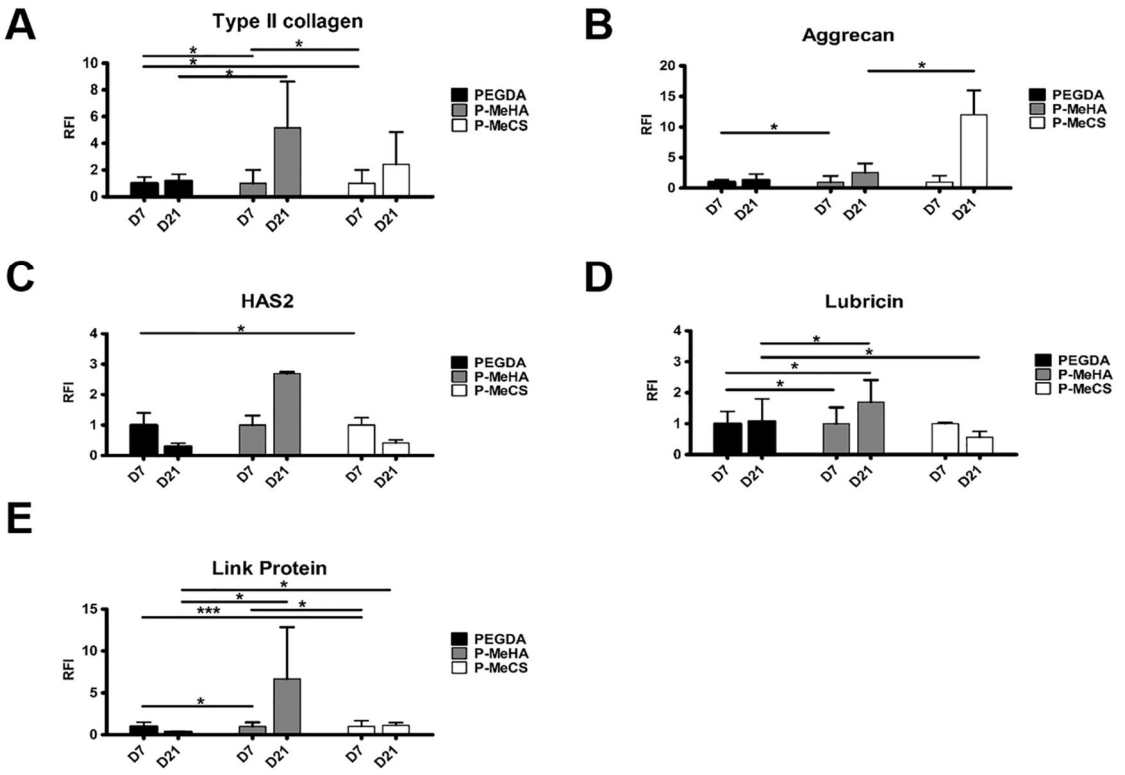


Figure 6.1 Relative gene expression level of Type II collagen **(A)**, Aggrecan **(B)**, HAS2 **(C)**, Lubricin **(D)**, and Link protein **(E)** from PEGDA, P-MeHA and P-MeCS. Within the relative fold induction, error bars represent the standard deviation on the mean for n=3. * $p < 0.05$, ** $p < 0.01$

2.3.5 *In vitro* histological analysis

Histological analysis was performed to assess the overall tissue quality after 3 weeks. Figure 6 shows the representative cross sections after 3 weeks of in vitro culture (Figure 7.1). The cell laden cryogels clearly indicated increasing cell proliferation by H&E staining, supporting the data obtained through DNA qualification (Figure 7.1.A-C). Furthermore, the addition of MeHA and MeCS had a greater impact on GAG secretion, showing a more intense Safranin-O staining compared to PEGDA cryogels (Figure 7.1.E-F).

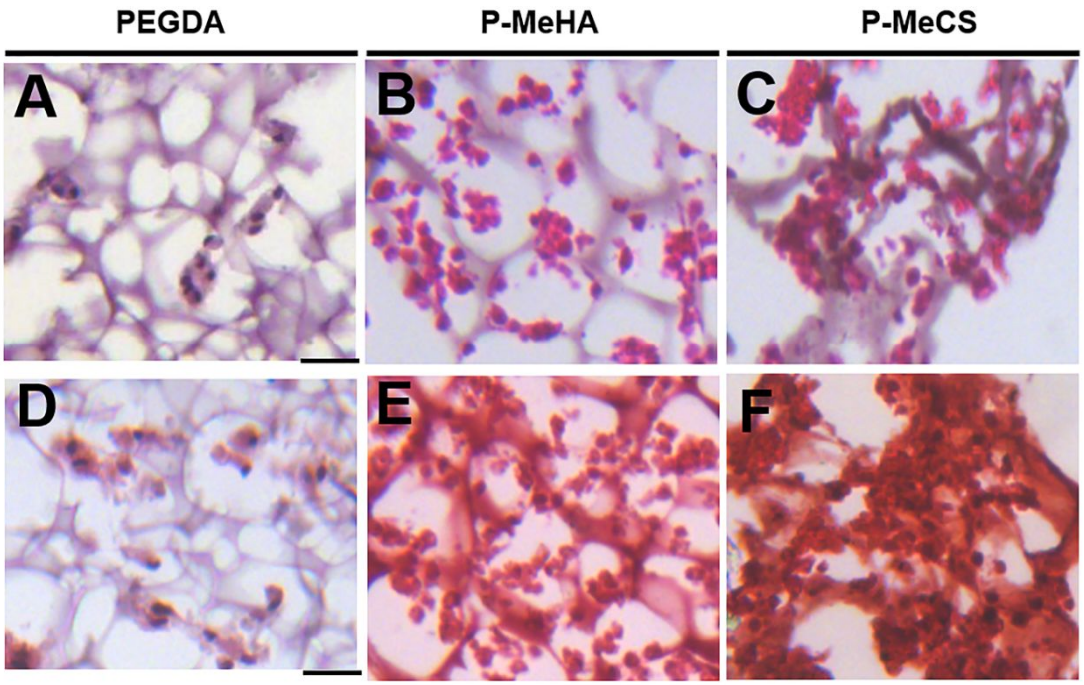


Figure 7.1 Histological analysis of *in vitro* engineered cartilage tissues. Hematoxylin and eosin staining of engineered cartilage tissues **(A-C)**, and Safranin-O staining **(D-F)** of cell laden on each cryogel group after 3weeks of in vitro culture. Hematoxylin and eosin staining visualizes the cellular morphology, while Safranin-O staining visualizes the deposition of GAGs. (Scale bar=100μm)

2.3.6 *In vivo* cartilage formation

Additionally, we examined *in vivo* response of chondrocytes in PEGDA, P-MeHA, and P-MeCS cryogels. No cellular inflammatory responses were observed after 6 weeks of subcutaneous implantation. H&E stained images showed that cells within the macroporous cryogel structures, were homogeneously distributed in all constructs (Figure 8.1.A-C) along with a rounded morphology of chondrocytes. Subsequently, the presence of proteoglycans was displayed by Safranin-O staining (Figure 8.1.D-F). The histological analysis showed greater Safranin-O staining in P-MeHA and P-MeCS constructs over PEGDA cryogel. In particularly, cartilage formation was visible with P-MeHA cryogel, which had a stiffer property on handling and more intense staining compared to others (Figure 8.1.E). Also, the HA and CS appeared to be partially degraded by ECM-specific enzymes. Next, the accumulation of the ECM components was also confirmed by immunohistological staining for aggrecan and collagen type type2 antibody which showed significant differences between PEG cryogels and ECM-based cryogels (Figure 9.1.A-F). Blue fluorescence specifies the cell of nuclei and green fluorescence indicates aggrecan and collagen type II antibody. By observing immunostained cryogel through confocal fluorescence microscopy, PEG cryogel did not

show intense green fluorescence (Figure 9.1.A-D). In contrast to PEG cryogel, ECM-based cryogel produced homogeneously concentrated cartilaginous ECM throughout scaffold as indicated by staining with aggrecan and collagen type II antibody. Especially, P-MeHA cryogel produced strong aggrecan and collagen type II network. Incorporation of HA may increase cellular activity by changing the scaffold mechanical properties and affect several potential mechanism [54].

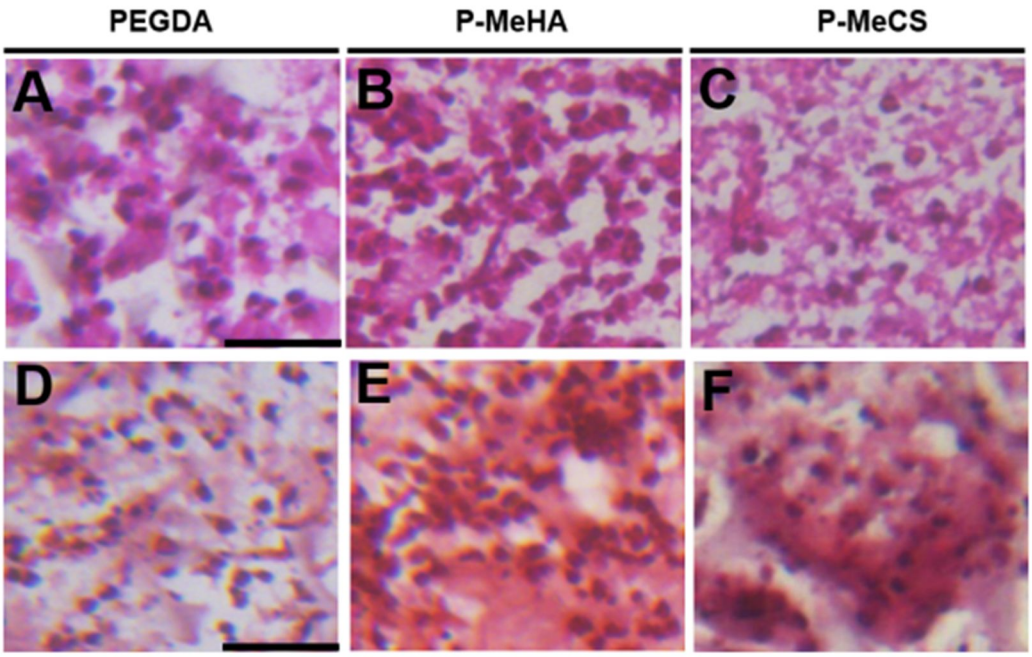


Figure 8.1 Histological analysis of in vivo engineered cartilage tissues. Hematoxylin and eosin staining **(A-C)**, and Safranin-O staining **(D-F)** of cell laden on each cryogel group after 6weeks of in vivo environment. Hematoxylin and eosin staining visualizes the cellular morphology, while Safranin-O staining visualizes the deposition of GAGs. (Scale bar=50μm).

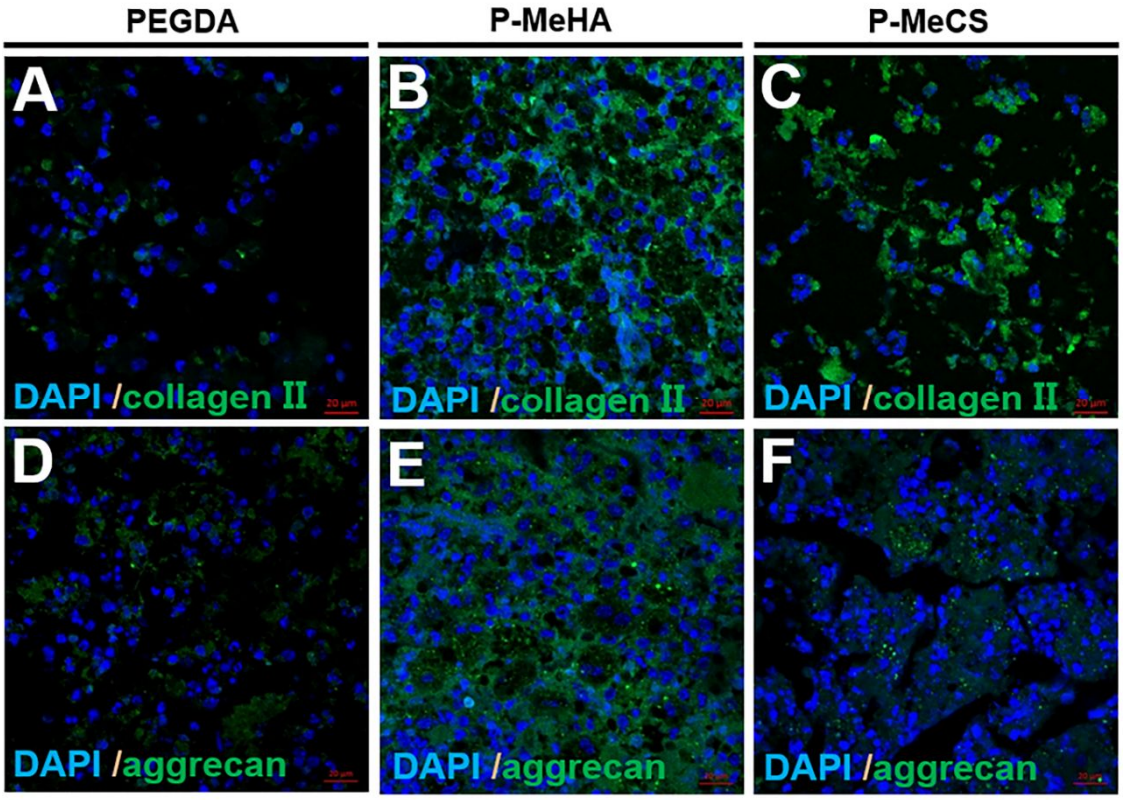


Figure 9.1 Result of collagen type II **(A-C)** and aggrecan **(D-F)** immunostaining with chondrocytes after 6weeks of in vivo environment. DAPI (blue) indicates cells and collagen type II (green) and aggrecan (green) demonstrates the presence of cartilaginous matrix. (Scale bar=20μm)

2.4 Conclusion

In this work, we examined ECM-based cryogels systems, which have characteristics of having high porosity, and tissue-like, elastic mechanical properties for the application of articular cartilage tissue engineering. Also, P-MeHA and P-MeCS cryogels increased the ECM matrix molecules deposition and enhanced the mechanical properties. Furthermore, cartilage-related genes were highly dependent on the types of cryogels as

shown by different responses to HA and CS environment. Subcutaneous implantation of ECM based cryogels in mice led to a formation of the densely interpenetrating network, supporting homogeneous cell distribution and cartilage specific ECM molecules. Moreover, the immunostaining analysis showed that the majority of the cartilage matrix was aggrecan and collagen type II. Our findings demonstrated that ECM based cryogels successfully support the accumulation of cartilage-specific ECM productions and can be one of the most promising applications in cartilage tissue engineering.

CHAPTER THREE: Gelatin based cryogels for cartilage tissue engineering

3.1 Introduction

Gelatin is derived from partial breakdown of natural triple-helical structure of collagen, thus, maintains bioactive features of collagen [55]. Since gelatin contains cell attachable signals, such as the Arg-Gly-Asp (RGD) sequence [56], it plays a crucial role in tissue engineering as a scaffold and have been widely used in several tissue engineering applications [57-59]. Especially, in the part of hydrogels, gelatin has been used for building cell scaffold [60, 61]. However, gelatin has insufficient mechanical properties to be used as a cell-scaffold and has physical temperature of it is below that of the physiological temperature required for cell culture [62]. To solve these drawbacks, gelatin was modified with chemical group to alter its physical properties via several methods [63-65]. Recent studies developed a new type of gelatin by incorporating isocyanate [66] or furfurylamine [61] to induce relatively high stiffness. Normal tissue cells showed different response to the stiffness of their substrate [67]. Typically, using methacrylate gelatin is favored due to its inexpensive and simple method [13, 68, 69]. Addition of methacrylate group to the amine-containing side groups of gelatin can form crosslinking using radical polymerization. GelMA hybrids were also suggested for diverse purposes [70], including tuning mechanical properties [71, 72] and providing environments similar to native tissue [71]. Additionally, to improve mechanical strength of gelatin hydrogel, other studies made hybrid gelatin scaffold with other polymer to

enhance mechanical properties [73]. In this work, gelatin methacrylate scaffold with hyaluronic acid/chondroitin sulfate were fabricated by cryopolymerization. We looked at the mechanical behavior, interior structure and cell viability, as well as the response of cellular behavior. After application of gelatin cryogels to mice through subcutaneous implantation for six weeks.

3.2 Materials and methods

3.2.1 Synthesis of gelatin- methacrylate

Methacrylated gelatins were synthesized as following. First, type A porcine skin gelatin (Sigma) was mixed at 10% (w/v) into phosphate-buffered saline (PBS; Gibco-BRL) at 60°C for 1hour. Then 8% (v/v) of methacrylic anhydride (Sigma) was added about 0.5ml/min and reacted at 50°C for 3hours to form GelMA solutions. After 5times dilution with additional warm (40°C) PBS to stop the reaction, mixtures were dialyzed against distilled water using 14kDa molecular weight dialysis for 1week at 40°C to remove salts and methacrylic acid. The solution was lyophilized and stored at -20°C until further use.

3.2.2 Fabrication of gelatin cryogels

With ammonium persulfate (APS; Sigma-Aldrich) as initiator agent and N,N,N',N' -tetramethylethylenediamine (TEMED; Sigma-Aldrich) as catalyst, Gelatin methacrylate cryogels were synthesized by free radical polymerization method. The premediated amount of Gelatin methacrylate, methacrylate hyaluronic acid and methacrylate

chondroitin sulfate was dissolved in water to the final desired concentration in the presence of 4% (v/v) APS and TEMED 0.2% (v/v), respectively. To start cryopolymerization, this polymer solution was put into cylindrical polyethylene mold and placed in the freezer (-20°C) for 20h. Interconnected and macroporous cryogels (diameter=8mm, height=4mm, and total volume = 200 µl) were synthesized by thawing. Prior to seeding cells, cryogels were sterilized with UV for 20 min and washed several times with PBS to remove unreacted residues. Rabbit chondrocytes were seeded onto three types of cryogels: GelMA, GelMA+MeHA (G-MeHA), GelMA+MeCS (G-MeCS). The samples were cultured in 1.5ml of chondrocyte medium (DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% HEPES, 1% MEM NEAA, 0.2% l-proline and 0.2% l-ascorbic acid) at 37°C in 5% CO_2 environment, and the medium was changed every 2 days for up to 3 weeks *in vitro*.

3.2.3 Physical characterization of gelatin based cryogels

Mechanical properties of Gelatin based cryogels were determined by Instron (5900S, USA). The specimens in each group were prepared as cylindrical blocks with size of 8mm in diameter and 4mm in height. Cell-free cryogel constructs were swollen in PBS for 24hours while cell-laden samples were fixed with 4% paraformaldehyde and tested the samples. The compressive modulus was calculated from linear slope of the stress-strain curve.

3.2.4 Statistical analysis

All Data are expressed as mean±standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA single factor) with * * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3.3 Results & discussion

3.3.1 Preparation and characterization of gelatin-based cryogels

Normal articular cartilage is comprised of chondrocytes and an extracellular matrix (ECM) which is consisted of collagen and proteoglycans [74]. Chondroitin sulfate and hyaluronic acid are involved as major component of proteoglycans. The GelMA (gelatin methacrylate) cryogel was thus prepared with methacrylated chondroitin sulfate and hyaluronic acid to mimic the natural cartilage extra cellular matrix (ECM) components. Generally, GelMA can form hydrogels with methacrylation varying from 20% to 80% [68]. In this study, GelMA cryogels were studied to assess their suitability as cell scaffolds in cartilage tissue engineering. To prepare cryogel solutions, MeHA (1% w/v) was mixed with GelMA (20% w/v) in 1:1 ratio to form HA-based cryogel (G-MeHA). Likewise, MeCS (10% w/v) was mixed with GelMA (20% w/v) in 1:1 ratio to form CS-based cryogel (G-MeCs). Cryo-polymerization was initiated via ammonium persulfate (APS) and N,N,N',N' -tetramethylethylenediamine (TEMED) mediated polymerization in freezing condition at -20°C. When cryogels do not perfectly form ice , a low degree of crystallinity was formed and stability of cryogels was decreased [59]. In this work, we fabricated gelatin based HA or CS cryogels with rabbit chondrocytes (Figure 1.1). The dried cryogel showed spongy-like morphology and swollen samples showed high water retension (Figure 2.1).

We further characterized the swelling property of each cryogels. We found that cryogel showed high swelling ratios (Figure 2.2) owing to interconnected macroporous structure. As a cell scaffold for tissue engineering, swelling ratio is also an important parameter. The porous interior has an influence on viability of seeded cells [42]. GelMA, G-MeHA and G-MeCS cryogels showed swelling ratios around 10. Additionally, we examined the interior morphology of cryogels. From SEM images, we observed that cryogel showed macroporous interconnected pore morphology and pore size of cryogels varied from tens to hundreds micrometers. Therefore, advantage of cryopolymerization can form macroporous structure using only water [59]. All these three types of cryogels, G-MeCS and G-MeHA, GelMA, showed porous interior (Figure 2.3). GelMA cryogel showed relatively larger pore diameters (102.74±41.66 μm) compared to other samples. G-MeHA (88.30±31.83 μm) and G-MeCS (89.01±31.26 μm) cryogel showed smaller pore size due to incorporation of methcarylated hyaluronic acid and chondroitin sulfate. The different temperature generated difference in pore sizes and inner structures in cryogels. During the fast cooling process, the density of hydrogels increased and formed smaller size crystals owing to heat transfer [59]. Furthermore gelatin solution concentration may have affected the pore size and interior morphology. When gelatin scaffolds were made from low gelation concentration (1%w/v), they showed high porosity and larger pore size whereas crystal growth was restricted in high gelatin concentrations [75].

We further characterized the compressive moduli of cryogels. In either, cell-laden or cell-free samples, there was no significant degradation but samples were softer than acellular samples. In addition, constructs were easy to handle. First, gelatin based

acellular cryogels were tested for compressive properties in wet conditions (figure 2.4.A). The compressive modulus of cryogels was significantly increased by addition of MeHA and MeCS. Especially, G-MeCS cryogel showed highest value of 37.64±8.74 kPa due to methcarylated chondroitin sulfate. The young's modulus of GelMA cryogel, 13.02± 0.801kPa was considerably smaller than those of G-MeHA (17.41±2.14 kPa) and G-MeCS cryogels. Although, small amount of MeHA and MeCS were incorporated, they showed significantly increased Young's modulus when compared to GelMA cryogels. Next, subcutaneously implanted samples were tested (figure 2.4.B). The largest change in Young's modulus was seen in G-MeHA, which increased by 116.83±19.57 kPa during 12weeks of culture. At the same time, G-MeCS cryogel showed significant increase (106.61 ±6.17kPa) when compared to GelMA (18.11±3.08 kPa). Also, values of cell-laden construct's modulus were higher than acellular construct in all groups. Especially, cell-laden G-MeHA group was 6.8-fold higher than cell-free. The cellular machanisms by MeHA are not celar, but studies hypothesized that the microscale phase separation of GelMA and MeHA helped facilitate distribution of the ECM [71]. In addition, other studies have shown that methacrylated hyaluronic acid hydrogel alone have low ability to promote larger-scale matrix distribution [76]. The newly accumulated matrix is expected to be more interconnected in samples with MeHA and MeCS, showing greater Young's modulus.

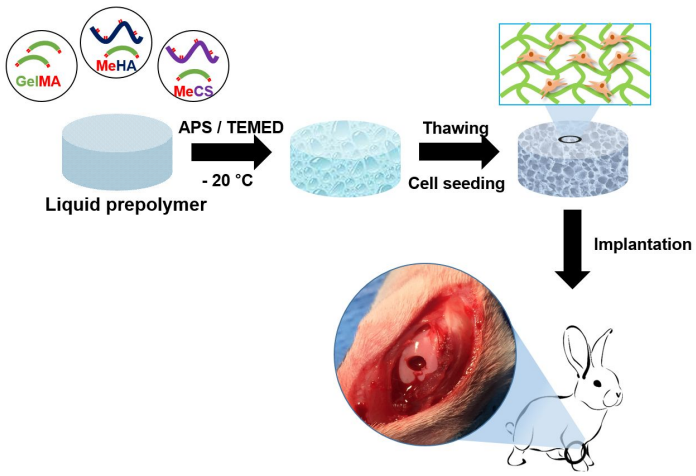
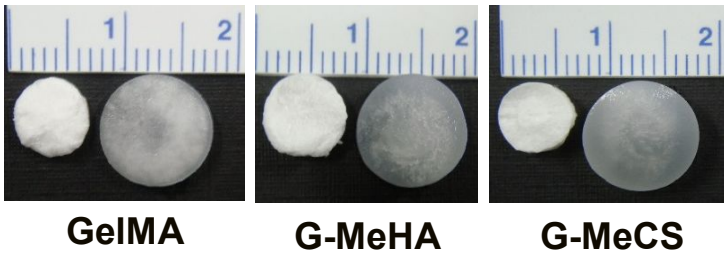


Figure 1.1 Overall schematic of research. Gelatin, chondroitin sulfate and hyaluronic acid were modified with acrylate groups to allow radical polymerization presence of radical initiators (APS and TEMED) in the frozen state. GelMA, G-MeHA and G-MeCS were mixed



with APS/TEMED and formed ice crystals at -20°C for 20hours.

Figure 2.1 Images of swollen (right) and lyophilized (left) cryogels. (GelMAA, G-MeHA and G-MeCS respectively)

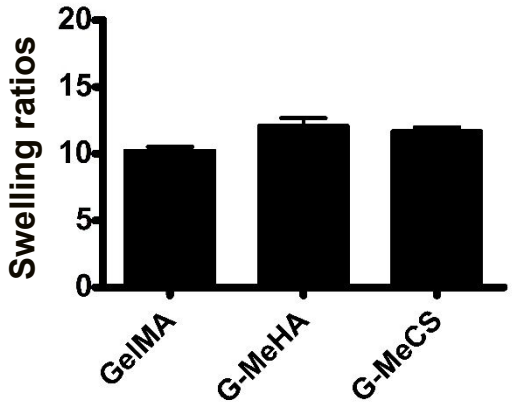
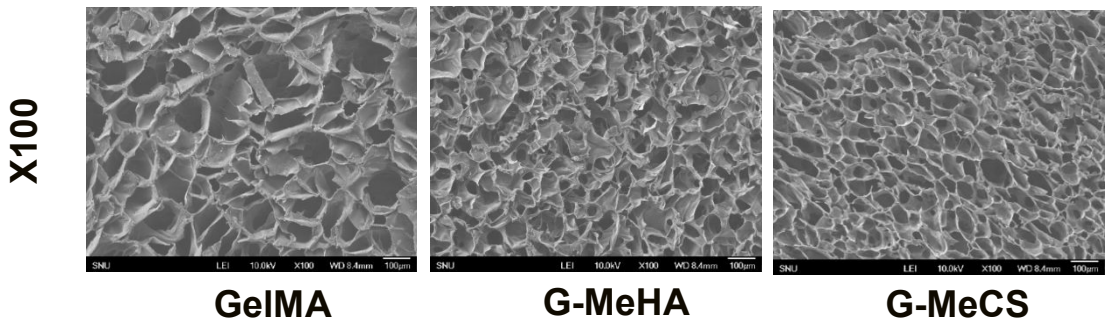
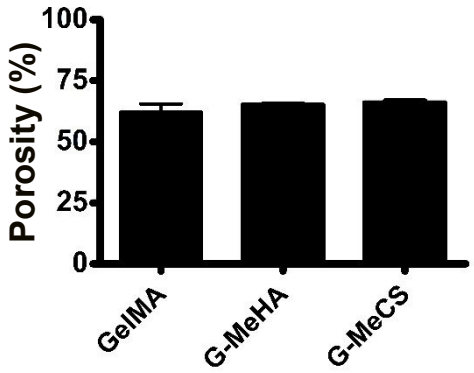


Figure 2.2 Swelling ratios of three types of swollen gelatin based cryogels in PBS for 24hrs. Similar swelling ratios was observed among the cryogels. Error bars represent the standard





deviation on the mean for n=4.

Figure 2.3 Scanning electron microscopy image of internal morphology of each cryogel. (Scale bar =10μm)

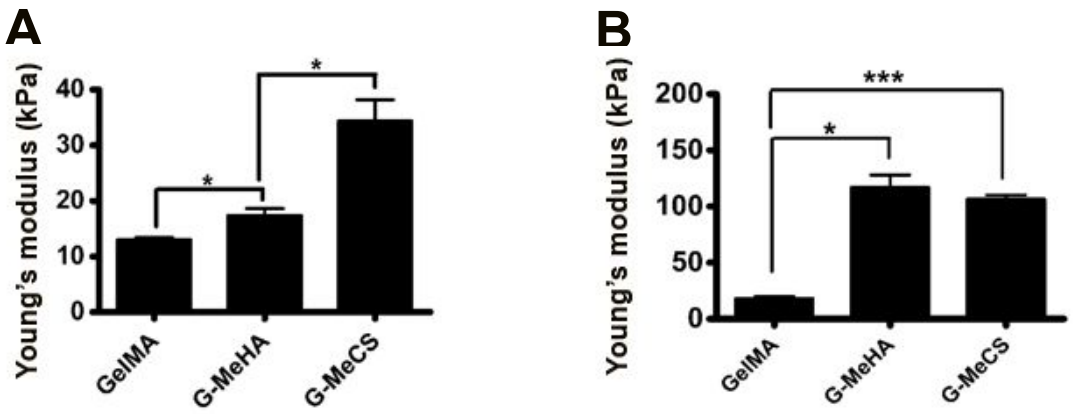
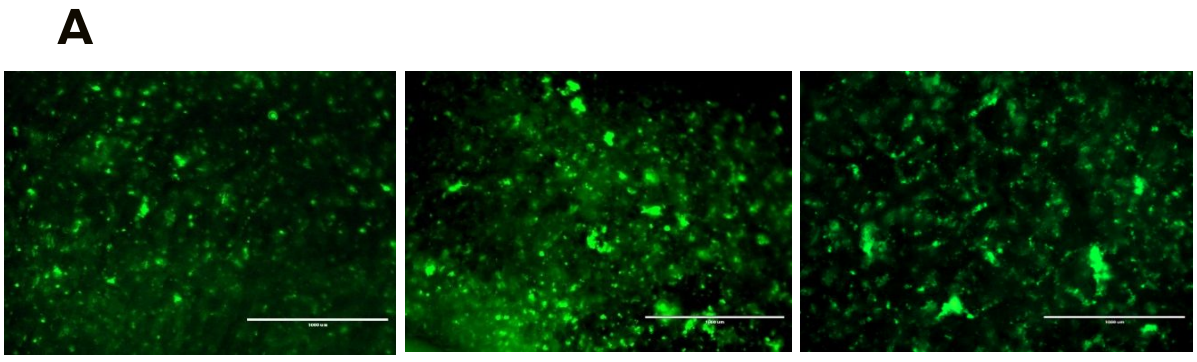


Figure 2.4 Young's modulus of cell-free gelatin based cryogels **(A)** and cell-laden cryogels **(B)**. GelMA, G-MeHA and G-MeCS were subcutaneously implanted for 12 weeks. Error bars represent the standard deviation on the mean for n=4.

3.3.2 *In vitro* cell viability on gelatin cryogels

To determine the biocompatibility of gelatin based cryogel, cell-laden samples were evaluated. Cell viability of rabbit chondrocytes in the presence of gelatin hybrid scaffold for 1day was analyzed using Live/Dead Cell viability/Cytotoxicity kit. The high viability (>90%) indicates that the gelatin based cryogels were biocompatible (Figure 3.1.B) similar to other reports [68]. Chondrocytes were uniformly distributed into the composite as shown with intense green fluorescence (live) instead of red (dead) (Figure 3.1.A). There was no significant difference of blending MeHA or MeCS on cell viability relative to GelMA scaffold.



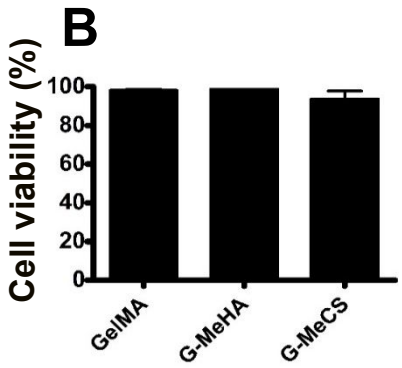


Figure 3.1 (A) Results of cell viability after 24hrous of *in vitro* culture and SEM images of internal fracture surface of cryogels with chondrocytes. In live/dead images (scale bar =1mm), living cells represent green and dead cells represent red. **(B)** Cell viability was calculated as (alive cells/ total cells) × 100%.

3.3.3 Effect of ECM-based cryogels on chondrocyte ECM synthesis

As a next step, DNA and GAGs contents were investigated as they are crucial for cartilage tissue regeneration. First, DNA contents at 3weeks were measured (provided in figure 4.1A-B). A significant increase in DNA content was observed during 3weeks between GelMA and G-MeHA and G-MeCS, respectively. The results showed that G-MeCS (0.698 ± 0.060ug/sample) had highest DNA contents while GelMA (0.458±0.0177 ug/sample) had lowest amounts. Newly accumulated GAG contents for each sample were shown in Fig.4B. A significant increase was found not only between

GelMA and G-MeHA but also in G-MeMA and G-MeCS. This is consistent with other reports showing that HA and CS can enhance ECM deposition in chondrocyte culture [77, 78]. The G-MeCS (41.94±1.045 ug/sample) cryogel showed highest GAG contents followed by G-MeHA (19.75±1.332 ug/sample) and GelMA (2.150±0.56 ug/sample). Similar increase of DNA and GAG was displayed indicating that ECM molecules may have promoted synthesis of cartilage-sepcific molecules. The incorporation of MeHA had fewer and less significant effects on chondrocytes than MeCS mixed cryogels. This could be because of higher concentration of MeCS. When MeCS were comparatively minor at 0.5%, the groups induced weak chondrocytes responses [79].

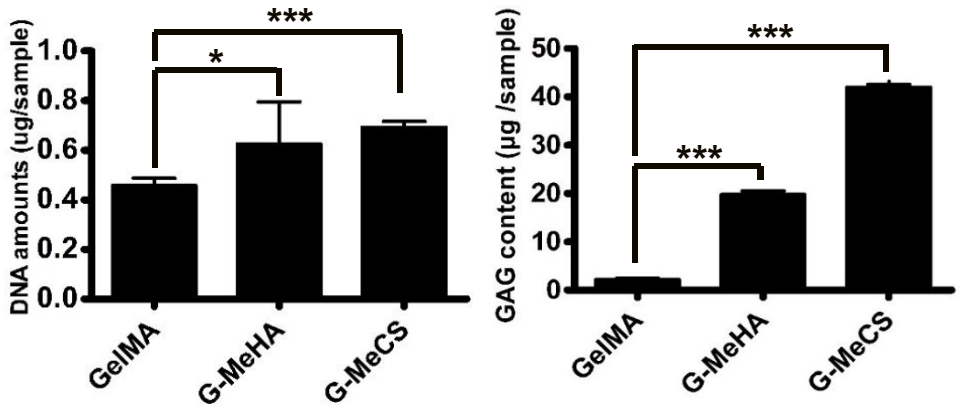


Figure 4.1 Result of biochemical analysis of the cryogel after 3weeks of *in vitro* cultivation. **(A)** DNA content and **(B)** GAG content was normalized to the dry weight of cryogel. Error bars represent the standard deviation on the mean for n=3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

3.3.4 Gene expression analysis of chondrocytes in gelatin based cryogels

To determine if the difference in scaffold composition mattered, we performed Real-time PCR analysis on culture of day7 and day21. Each cryogels at day21 were normalized to samples from day7 (figure 5.1). Firstly, we evaluated the expression of cartilage related genes aggrecan, PRG4, HAS2, Link protein, type X collagen and type II collagen. Among them, aggrecan is negatively charged biopolymer that forms supermolecular complexes with hyaluronic acid and these complexes provide high osmotic properties that resist compressive load [80, 81]. type II collagen and type X collagen forms fibril structure which play a key role in structural stabilization [82]. Especially, type II collagen is essential for cartilage tissue owing to high contents of hydroxyl-lysine as well as glucosyl residues which mediate the interaction with proteoglycans, another typical component of cartilage [82, 83]. Gelatin containing HA showed a significant increase in collagen type X, HAS2, link protein and collagen type 2 at day 21 as compared to the GelMA group at day 21, while Gelatin hydrogels without extracellular matrix molecules, did not show an increase in type X collagen, aggrecan, PRG4 and HAS2 (figure 5.1). Aggrecan gene showed that a constant level of expression in G-MeHA from D21 when compared to D7. Type II collagen gene expression was more evident in all types of cryogels between D7 and D21. Type II collagen expression showed 10-fold increase in GelMA scaffold compared to controls at day 7, 7-fold increase in G-MeHA and 550-fold increase in G-MeCS cyogels. Except for type II collagen and link protein, type X collagen, aggrecan, PRG4 and HAS2 expression was downregulated in GelMA groups when compared day 21 to day 7. On the other hand,

G-MeCS groups showed strong upregulation in all types of cartilage related gene. According to other studies, addition of chondroitin sulphate C contribute to the accumulation of type II collagen during 3D culture of chondrocytes cells [78]. Moreover, CS stimulates the chondrocyte metabolism, leading to synthesis of collagen and proteoglycan [84]. Lastly, the presence of MeHA and MeCS resulted in highly upregulated type II collagen, HAS2, link protein and type X collagen gene expression.

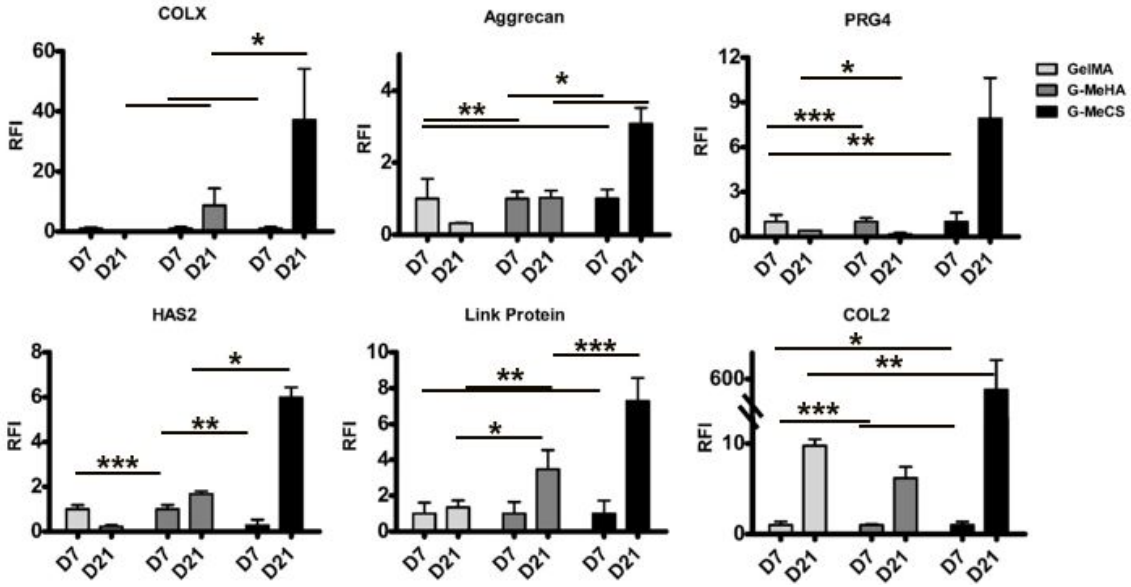


Figure 5.1 Relative gene expression level of type X collagen, aggrecan, PRG4, HAS2, link protein and type II collagen at 1week and after 3weeks culture. Error bars represent the standard deviation on the mean for n=3.

3.3.5 *In vitro* histological analysis

Next, we stained Hematoxylin/eosin and Safranin-O for staining which were widely used in histology. Generally, hematoxylin stains for cell nuclei (blue) while eosin stains cell membrane and cytoplasm (pink). As seen in figure 6.1.A-C, H&E staining showed that cells were homogenously distributed throughout all types of cryogels. Rabbit chondrocytes and gelatin scaffold were clearly distinguishable owing to intense or dim color. Furthermore, chondrocytes placed in scaffolds had rounded appearance. The glycosaminoglycans within cell containing constructs were evaluated by Saf-O staining. The intensity of Safranin-O staining is directly proportional to the proteoglycan contents, which is consisted of core proteins and glycosaminoglycans. Figure 6.1.D-F displays cross section of samples at day 21. Due to different composition, such as MeHA and MeCS, these samples show more intense Saf-O staining compared to GelMA cryogels. Histological results indicated that the G-MeCS cryogels degradaed faster than G-MeHA which may allow for the deposition and enhanced distribution of newly synthesized ECM proteins. The timing of scaffold degradation is important because rapid degradation may induce insufficient accumulation of ECM proteins [85].

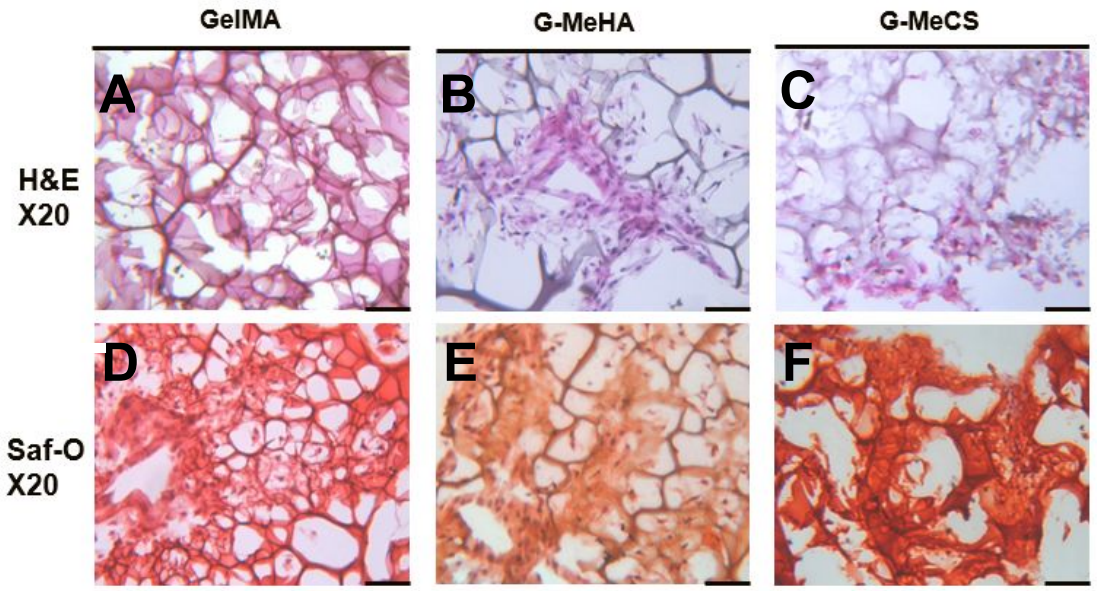


Figure 6.1 Results of hematoxylin and eosin staining **(A-C)**, and Safranin-O staining **(D-F)** of cell laden on each cryogel group after 3weeks of *in vitro* culture. Hematoxylin and eosin visualizes cellular morphology, Safranin-O staining visualizes deposition of GAGs. (Scale bar=100μm)

3.3.6 *In vivo* histological analysis

After 6weeks of subcutaneous implantation, the cell-laden scaffold formed opaque cylindrical shape and maintained their original shape. As expected, HA and CS induced groups showed more rigid hardness and maintained cartilage-like apperance compared to gelatin scaffold. H&E stained images showed that cells were homogenously distributed through all types of scaffold (figure 7.1). Additionally, G-MeHA and G-

MeCS cryogels showed strong expression of aggrecan and collagen type II displaying intense green fluorescence (figure 7.2). Lastly, the culture with the addition of ECM molecules showed promising staining results compared to ones without.

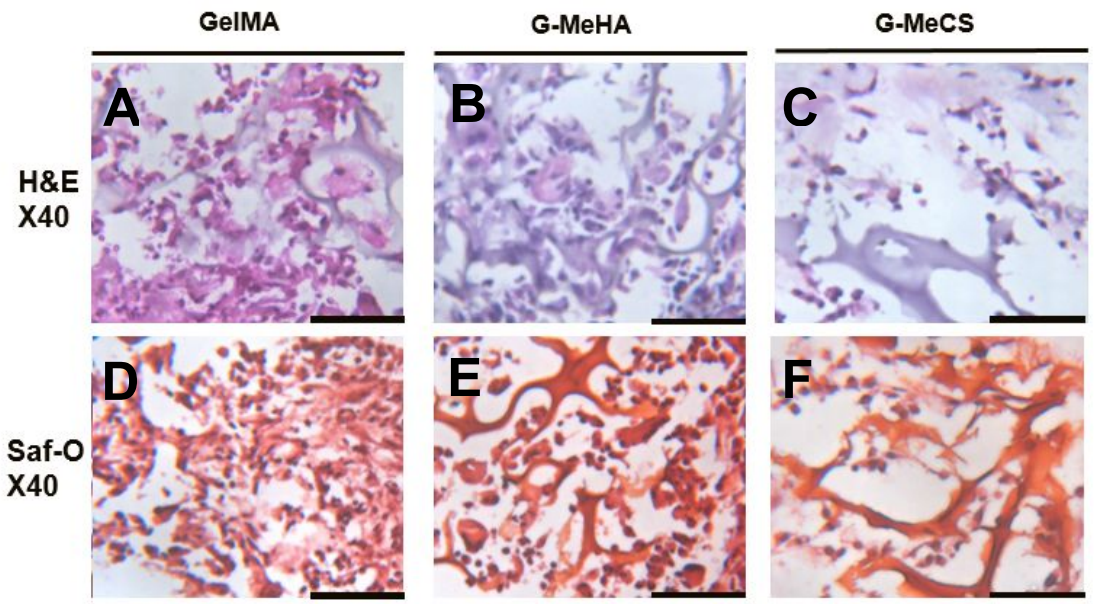


Figure 7.1 Results of hematoxylin and eosin staining **(A-C)**, and Safranin-O staining **(D-F)** of cell laden on each cryogel group after 6weeks of *in vivo* environment. Hematoxylin and eosin visualizes cellular morphology, Safranin-O staining visualizes deposition of GAGs. (Scale bar=50μm)

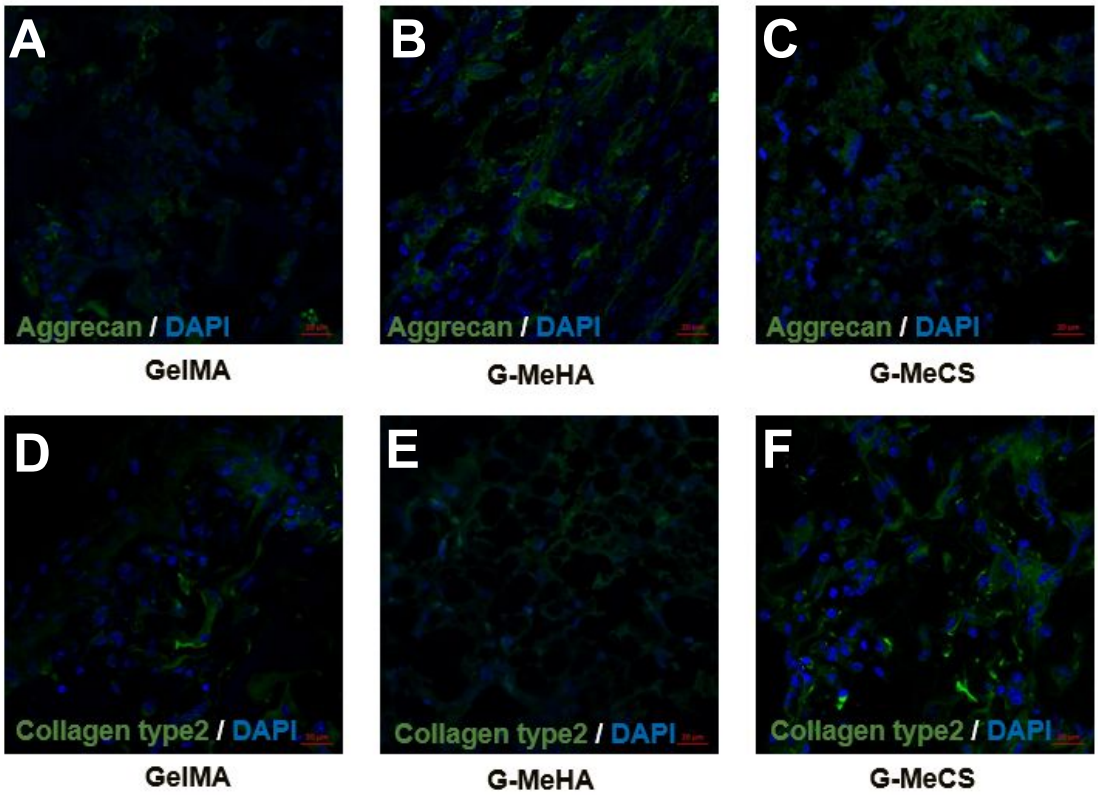


Figure 7.2 Result of aggrecan **(A-C)** and collagen type II **(D-F)** immunostaining with chondrocytes after 6weeks of in vivo environment. DAPI (blue) indicates cells and collagen type II (green) and aggrecan (green) demonstrates the presence of cartilaginous matrix. (Scale bar=20μm)

3.4 Conclusion

In this study, the effect of scaffold components, methacrylated HA and CS, on chondrocytes was evaluated. We fabricated the gelatin cryogels, which had suitable macroporous strucutre and tissue-like elastic mechanical properties via freeze-drying method. Chondrocytes grown in G-MeHA and G-MeCS showed significant differences in cartilage related gene expressions compared to GelMA. Additionally, with MeHA and MeCS, cryogels exhibited higher accumulation of GAGs and DNA contents than

control samples. Immunostaining analysis confirmed that different composition of subcutaneously implanted, gelatin based scaffolds can produce chondrogenic molecules such as aggrecan and collagen type II. Therefore we demonstrated that scaffold materials play an important role in regulating cellular response in chondrocytes and that components should be considered preferentially to form stable and strong cartilage tissues.

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Table 1.1 Primer list

Gene	Sequence
GAPDH	F: TCA CCA TCT TCC AGG AGC GA R: CAC AAT GCC GAA GTG GTC GT
Aggrecan	F: CCT TGG AGG TCG TGG TGA AAG G R: CCT TGG AGG TCG TGG TGA AAG G
HAS2	F: TTT CTT TAT GTG ACT CAT CTG TCT CAC CGG R: ATT GTT GGC TAC CAG TTT ATC CAA ACG G
Type II collagen	F: TTC ATG AAG ATG ACC GAC GA R: GAC ACG GAG TAG CAC CAT CG
Lubricin	F: TTA CCG ATG TCT GGG GCA TAC CTT C R: TGG GCA GTG ATA TAG CTG AGG TGA CC
Link protein	F: AGG CTG TAC AAC AGA GCA CC R: AAA CAA GTC CCG GCT CTC AG

요약(국문초록)

연골 조직 공학을 위한 세포외기질과 합성/천연 고분자를 응용한 크라이오젤 합성

냉동 겔화 (cryogelation) 기반의 크라이오겔은 세포 치료, 조직 엔지니어링, 약물 전달 및 스캐폴드 기반 면역 치료를 포함하는 임상적용에 유용한 것으로 알려져 있다. 냉동 겔화의 과정은 용액내의 순수한 얼음 결정이 형성되는 것으로부터 시작된다. 이후에 얼음결정들이 용해되고 기송의 네트워크만 남게 된다. 본 연구에서는 폴리에틸렌 글리콜 디아크릴레이트와 젤라틴 기반에 세포외기질의 종류인 콘드로이틴 황산염 (chondroitin sulfate) 히알루론산 (hyaluronic acid)을 함께 가교시켜 크리오겔 내에서 고유 생리 활성을 도입 및 유지 할 수 있도록 하였다. 토끼 연골세포를 크라이오겔에 적용시킨 결과, 콘드로이틴 황산염과 히알루론산이 첨가됨에 따라 유전자와 (DNA) 글리코사미노글리칸 (glycosaminoglycan)의 양이 증가한 것을 확인하였다. 또한 중합효소연쇄반응을 통해 콘드로이틴 황산염 이 함유된 젤라틴 기반의 실험 군 에서, type II collagen, type X collagen, aggrecan, HAS2, PRG4, Link protein 등이 가장 높은 유전자 발현 정도를 보였다. 위

결과를 토대로 콘드로이틴 황산염과 히알루론산이 첨가된 크라이오겔이 연골 조직 공학 응용에 널리 쓰일 수 있음을 증명하였다.

주요어: Cryogel, Hyaluronic acid, Chondroitin sulfate, Cartilage Tissue engineering, Poly (ethylene glycol) diacrylate, Gelatin

학번: 2014-20595