공학석사 학위논문

Preparation and Characterization of Porous Poly (Vinyl Alcohol) (PVA) Hydrogel filled with Multifunctional Extracellular Components for Cartilage Regeneration

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Abstract

Preparation and Characterization of Porous Poly (Vinyl Alcohol) (PVA) Hydrogel filled with Multifunctional Extracellular Components for Cartilage Regeneration

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Articular cartilage has a very limited self-repair ability due to its avascular structure. Cartilage defect causes several dysfunctions as decreased load resistance and lubrication during joint motions. Poly (vinyl alcohol) (PVA) hydrogel has been proposed as a non-degradable and biocompatible FDA-approved synthetic biomaterial. In this study, we fabricated PVA sponge filled with bioactive components and applied them for cartilage repair application. Photopolymerizing chondroitin sulfates (CS) or hyaluronic acid (HA) were injected into macroporous PVA sponge with chondrocytes to fill the pores. Our study showed that CS enhanced the extracellular

matrix accumulation and chondrocyte-related gene expressions. Moreover, HA increased the expression of genes involved in the lubrication process of articular cartilage. Overall this study demonstrates durable PVA hydrogel incorporated with bioactive components can be further utilized for functional cartilage tissue engineering.

Keywords: PVA (Poly Vinyl Alcohol), Hydrogel, Hyaluronic Acid, Chondroitin Sulfate, Chondrocyte, Cartilage Tissue Engineering

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Chapter ONE: The Scientific Background and Research Progress

1.1 Cartilage tissue engineering

Hyaline cartilage, a typical structure of cartilage in our body, plays a critical role in reducing friction and maintaining wear-resistant property of articulating joint [1]. However, hyaline cartilage is easily damaged due to various causes such as injuries by repeated stress loading and degenerative joint diseases by natural aging or genetic mutation [2, 3]. Despite the susceptibility of impairment in hyaline cartilage, anatomical architecture that appears to have no vascular and lymphatic systems limits the native tissue regeneration [3]. Ongoing therapeutic reconstruction of cartilage involves autologous chondrocyte implantation, osteochondral grafts, and microfracture techniques [4]. (Figure 1.1) However, those methods still impede cartilage repair regarding organization and functions [4]. Because of these drawbacks, recent studies pay more attention to tissue engineering that seeks cartilage regeneration via incorporation of cells, scaffolds and growth factors [5]. Cartilage tissue engineering has shed a light on the treatment of damaged cartilage with advantageous progress, yet particular improvement in the regeneration of functional tissue is remaining.

Intricate and discrete mechanical properties of articular cartilage arise from its layered framework with different contents. Typically, articular cartilage is comprised of chondrocytes and abundant extracellular matrices (ECMs) that are primarily built up by type II collagen and proteoglycans [6]. Hierarchically, four distinct layers compose articular cartilage and these four layers are clearly distinguishable from each other by configuration of collagen fiber and proteoglycans. Progressing from the very uppermost layer to the lowermost layer just above the bone, the superficial layer has horizontally ordered fibers, the middle layer has disorganized fibers, the deep layer has vertically ordered fibers to the bone surface and the last calcified layer has few or no alignment but rather mineralized. Contrarily, the amount of proteoglycan is the least in the uppermost superficial layer and rises in deeper layer. Not only that, depth of layers, ECM composition, and morphology of cells also vary in each layer [7]. The collagen alignments in different layers resulted in a difference of tensile and shear characteristics. However, distribution of proteoglycans in different layers led to compressive feature that differs from each layer [8, 9]. Particularly, the deep layer is 10 ~ 20 times stiffer than the superficial layer. Along with complicated functionality of articular cartilage structure, collagen fiber

arrangement restrains the tissue from swelling, whereas negatively charged proteoglycans and cartilage tissue with low permeability facilitate swelling of the tissue via water retention [10]. The water retention capability of the tissue is decisive to withstand physical pressure in dynamic loading condition [11]. Combining the role of collagen network and proteoglycans, cartilage possesses strong load bearing and low frictional coefficient competence [12, 13]. There are many studies that demonstrate mechanical and compositional characteristics of native cartilage tissue in general. However, not many have proved elaborate architecture and role of native cartilage raised in cartilage tissue engineering.

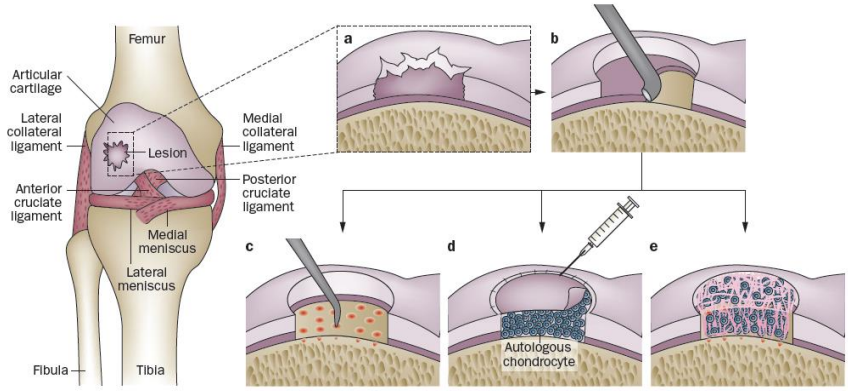

Figure 1.1 Cartilage regeneration techniques. a | A full-thickness focal chondral lesion. b | The lesion is debrided to ensure healthy, stable margins for integration of the host tissue with the neotissue. c | Microfracture. Channels are created using a 45° awl, spaced 3–4mm apart, and 3–4mm deep to penetrate the subchondral bone, allowing MSCs to migrate from the marrow to the cartilage defect. d | ACI. The debrided lesion is filled with 12–48 million autologous chondrocytes and covered with a periosteal flap or mixed collagen type I and type III membrane. e | MACI. The autologous chondrocyte population is expanded in vitro and then seeded for 3 days onto an absorbable 3D (collagen types I and III or hyaluronic acid) matrix prior to implantation. The cell-seeded scaffold is then secured into the lesion with fibrin glue. Abbreviations: ACI, autologous chondrocyte implantation; MACI, matrix-assisted autologous chondrocyte implantation; MSC, mesenchymal stem cell. [14]

1.2 Polyvinyl alcohol (PVA) and Polyethylene glycol diacrylate (PEGDA) hydrogel

Poly (vinyl alcohol) (PVA), hydrolyzed form of poly(vinyl acetate), has been widely utilized as cartilage-replacement materials due to its in superior biocompatibility, non-toxicity and proper mechanical strength [15-17]. (Figure 1.2) Furthermore, with the approval of FDA, abundant biomedical applications of PVA have been developed such as artificial blood vessels, contact lens, surgical sponges and implantable medical materials [18, 19]. However, PVA exhibits high hydrophilicity with the hydroxyl group (-OH) that causes poor adhesion to native cartilage [16]. Moreover, cell encapsulation in PVA scaffold has not been studied widely. Therefore, we have introduced PEG-based photopolymerizable macromeres [20] with methacrylated bioactive ECM polysaccharides such as chondroitin sulfate (CS) and hyaluronic acid (HA). Photocrosslinkable hydrogel is utilized in various studies because of its homogenous and highly dense cell encapsulation [21]. Also, ease of shape and dimension modulation is another advantage of photocrosslinkable hydrogel widely applied in tissue engineering [22].

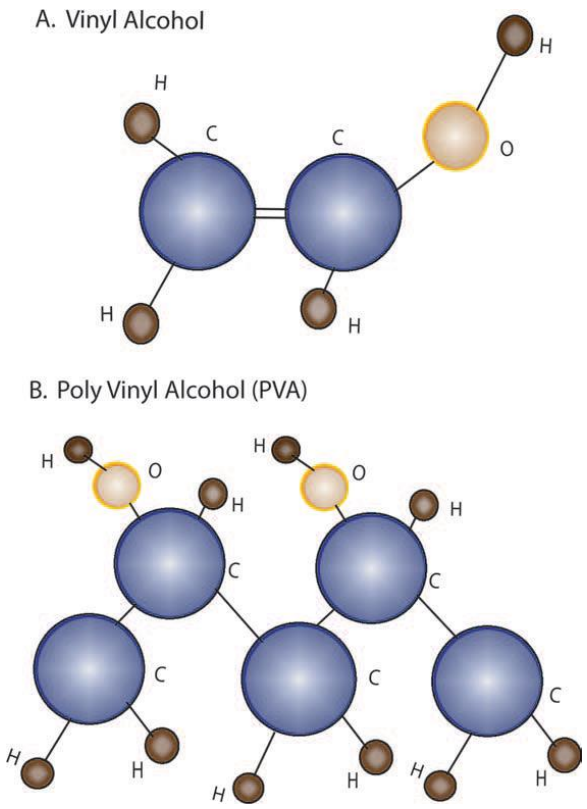

Figure 1.2 A: The structure of vinyl alcohol is shown. B: PVA is synthesized by the hydrolysis of polyvinyl acetate. The structure of PVA is shown in this figure. Typical levels of hydrolysis are from 80% to greater than 99%, with PVA hydrogels formed from nearly fully hydrolyzed forms. [18]

1.3 Research aims

This study examined the fabrication of porous PVA sponge filled with ECM-based photopolymerizing hydrogel for effective cartilage regeneration. In addition to the hydrogel characterization, cellular responses were investigated. Photopolymerization of ECM-based macromers enabled chondrocytes encapsulation into PVA sponge. ECM-based microenvironments promoted GAGs accumulation and collagen secretion by encapsulated chondrocytes. Furthermore, cartilage-related gene expressions were up-regulated in ECM-based hydrogels compared to hydrogels without ECM molecules. In brief, we have hypothesized that combining PVA with photocrosslinkable PEGDA supplemented with ECM compound would have remarkable potential for cartilage tissue engineering.

Chapter TWO: Fabrication and Characterization of PVA hydrogel filled with Multifunctional ECM components

2.1 Introduction

Articular cartilage mainly consists of extensively secreted extracellular matrix (ECM) components by chondrocytes. Cartilage specific ECM is composed of collagen, proteoglycans, and noncollagenous proteins [23]. Numerous studies have been conducted to overcome the limitation of cartilage repair utilizing biomimetic scaffolds [24-28]. Scaffold-based tissue engineering for articular cartilage is a promising strategy for autologous cell delivery with biological favorable materials. For synergistic effect of the scaffold material-driven tissue regeneration with cells, biocompatibility and the ability to induce dynamic synthesis of cartilage ECM should be thoroughly considered. Thus, this study aimed to combine macroporous PVA and photocrosslinkable hydrogel with chondrocyte encapsulation to establish an optimal scaffold for cartilage regeneration.

2.2 Materials and methods

2.2.1 *Chemicals and reagents*

All chemicals and reagents are purchased from the vendors as mentioned and stored following the manufacturer's instruction: PVA sponge (Medtronic Xomed), Poly (ethylene glycol) diacrylate (PEGDA; MW 3400; Alfa Aesar), Sodium hyaluronate (HA; Lifecore Co.), Chondroitin sulfate (CS; MW 20,000 ~ 40,000; Tokyo Chemical Industry), Glycidyl methacrylate (GMA; Sigma-Aldrich), Photoinitiator (Irgacure 2959), Phosphate buffered saline (PBS; Gibco), Fetal bovine serum (FBS; Gibco), Dulbecco's modified Eagle's medium (DMEM; Gibco), Penicillin/Streptomycin (Pen-Strep; Gibco), 4-(2-hydroxyethyl)-1-piperazaineethanesulfonic acid (HEPES; Gibco), Non-essential amino acid (NEAA; Gibco), L-ascorbic acid (Sigma-Aldrich), L-Proline (Sigma-Aldrich), Deuterium oxide (Sigma-Aldrich), Live/dead Cell viability/cytotoxicity kit (Molecular Probes, L-3224), Quanti-iT^{TM} PicoGreen dsDNA Assay Kit (Invitrogen), Papainase solution (Worthington Biomedical).

2.2.2 *Chondrocyte isolation and culture*

Chondrocytes were isolated and cultured as previously described [29]. In brief, articular cartilage fragments were collected from articular cartilage of New Zealand white rabbits (Koatech Laboratory Animal Company). Cartilage pieces were washed with PBS three times and digested with 0.2% (w/v) collagenase type II (Worthington Biomedical) in DMEM supplemented with 1% (v/v) of Pen-Strep for 18 hours. Isolated chondrocytes were washed and cultured in DMEM medium containing 10% (v/v) FBS, 1% (v/v) Pen-Strep, 1% (v/v) HEPES, 1% (v/v) NEAA, 0.2% L-proline, and 0.2% L-ascorbic acid at 37°C in 5% CO_2 environment. Culture medium was changed every 2 days.

2.2.3 *Methacrylation of chondroitin sulfate and hyaluronic acid*

Methacrylation of chondroitin sulfate (MeCS) and hyaluronic acid (MeHA) were conducted from carboxyl functional group of both polysaccharide structures with GMA. The reaction followed the previously described synthesis procedure [30]. CS was dissolved in PBS at 10% (w/v) and 10% (v/v) GMA was added drop wisely. The mixture was then reacted for 11 days with continuous stirring. The resulting solution was then dialyzed against deionized water with

1000MW dialysis membrane for 2 days. The dialysis water was changed 3 ~ 4 times per day. The resulting final solution was frozen at -80°C and lyophilized. Methacrylated CS was stored at -20°C until usage. For HA, same protocol was applied. HA was dissolved in PBS at 1% (w/v) and 2% (v/v) GMA was added. The mixture was reacted for 8 days with vigorous stirring. The remaining dialysis and lyophilization procedure followed the same procedure as CS methacrylation.

2.2.4 *Photoencapsulation and incorporation of ECM-based hydrogel into PVA sponge*

We fabricated PVA sponges filled with bioactive components, chondroitin sulfates (CS) or hyaluronic acid (HA). Photopolymerizing chondroitin sulfates (CS) or hyaluronic acid (HA) were injected into macroporous PVA sponge with rabbit chondrocytes to fill the pores. The hydrogel without CS or HA was fabricated with PEGDA dissolved in PBS at 20% (w/v). ECM-based hydrogels were prepared with MeCS and MeHA dissolved in PBS at 10% (w/v) and 1% (w/v) respectively. This pre-gel polymer solution was mixed in 1:1 ratio for each experimental group; PEGDA+MeCS (PVA-CS) and PEGDA+MeHA (PVA-HA). Rabbit chondrocytes were mixed with

the polymer solution at a concentration of 1×10^6cells/construct. Photoinitiator (Irgacure 2595) was also added to the polymer solution at 0.05% (w/v) concentration. Photocrosslinkable macromer solution was slowly injected into PVA sponge placed in cylindrical polyethylene mold. After the pre-gel solution fills the pore of macroporous PVA sponge, PVA sponge was exposed to UV light ($3.5mW/cm^2$) for 5 minutes. The construct was then removed from the mold and culture at 37°C in 5% CO_2 in chondrocyte medium. Culture medium was changed every 2 days for 3 weeks in vitro. Rabbit chondrocytes were also seeded onto PVA sponge directly without photo-encapsulation. Prior to the cell seeding, PVA sponges were sterilized in UV overnight.

2.2.5 *Swelling ratio and mechanical analysis*

Hydrogels were swelled in PBS for 24 hours. The wet weight of the hydrogels was measured after wicking the extra PBS on the surface of hydrogels by weighing paper. The weighed hydrogels were then freeze-dried overnight for the measurement of dry weight. Swelling ratio of the hydrogels was calculated following the equation below.

$$\text{Swelling Ratio (Q)} = \frac{wet\ weight\ of\ equilibrated\ hydrogel\ in\ PBS}{dry\ weight\ of\ hydrogel\ after\ lyophilization}$$

For the mechanical test, hydrogels were swollen in PBS overnight. Instron 5966 (Instron Corporation) with 100KN maximum load was used for measuring Young's modulus. Before the measurement, diameter and height of each hydrogels were gauged by caliper for further calculation. The pressure was applied to the hydrogel surface until 40% of hydrogel strain with the rate of 1.5mm/min. Young's modulus was obtained from the linear region of stress-strain curve.

2.2.6 *Cell viability*

Viability of chondrocytes was determined by Live/Dead Cell viability/cytotoxicity kit following the manufacturer's protocol (Molecular Probes, L-3224). Images were collected by Zeiss 720 laser scan confocal microscope. Cellular viability was quantified by Image J software.

2.2.7 *Statistical analysis*

All data are presented as mean ± standard error of the mean (SEM). Statistical significance between groups were determined by Student's t-test with *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

2.3 Results

2.3.1 Preparation and characterization of macroporous PVA sponge with photopolymerizing ECM-based hydrogels

Porous PVA sponges were incorporated with ECM-based photopolymerizing hydrogel. (Figure 2.1) For ECM-based hydrogel fabrication, CS and HA are reacted with GMA for methacrylation. GMA reacted with hydroxyl, carboxyl, and sulfate group of CS and HA to become reactive vinyl groups for free radical polymerization [31]. Methacrylation on CS (MeCS) and HA (MeHA) were confirmed by performing ^{1}H nuclear magnetic resonance (^{1}H NMR). ^{1}H-NMR spectra of MeCS indicated acrylate group peaks at 6.151ppm and 5.716ppm. Acrylate group peaks in MeHA were observed at 6.157ppm and 5.710ppm. (Figure 2.2) Therefore, we confirmed the existence of acrylate functional group onto CS and HA backbone. For incorporation of ECM-based photopolymerizing hydrogel into PVA sponge, MeCS and MeHA was mixed with PEGDA and injected into sterilized PVA sponge. Pre-gel photocrosslinkable solution filled the pores of macroporous PVA sponge and then exposed to UV light for photopolymerization. Figure 2.3 shows the gross images of highly porous PVA sponges non-swelled and swelled. The diameter of the PVA sponge was 5mm

and the height was 2.75 ± 0.5mm. (Figure 2.3)

Acellular samples were characterized for their equilibrium swelling ratio (Q) and Young's modulus (n=3). PVA sponge without any polymer cross-linked indicated 12.4±0.44 swelling ratio while three other PVA hydrogels photo-crosslinked with polymers exhibited lower swelling ratio. PVA-CS hydrogel showed the lowest swelling ratio with 7.75±0.86. PVA-HA and PVA-PEG hydrogel indicated 9.32±0.27 and 9.73±0.63 swelling ratio, respectively. (Figure 2.4 Top)

Young's moduli of acelluar samples were also examined. Compression test was conducted until 40% strain of the hydrogel and the samples with PVA sponge did not reach the breakage and maintained their cylindrical shape till the 40% strain point. However, samples without PVA sponge showed partial or complete breakage before 40% of compressive strain was applied. All samples were swollen to the equilibrium state before the mechanical test. PVA sponge indicated the lowest mechanical property while photo-crosslinking to the PVA sponge enhanced the modulus by filling the pores with the polymer cross-linked network. Increase of Young's modulus was at least 1.5 times compared to PVA sponge. PVA sponge showed 36.38±1.63kPa while the highest Young's modulus

was shown by PVA-PEG as 101.18±4.42kPa. (Figure 2.4 Below) Photopolymerized hydrogels without PVA incorporation displayed lower mechanical property compared to the hydrogels with PVA. Specifically PVA-PEG and PVA-CS displayed significantly increased Young's modulus than PEG and CS hydrogels. PVA-HA indicated higher modulus than HA without statistical significance. Young's moduli of hydrogels without PVA were 42.43±3.28kPa, 46.48±5.79kPa, and 45.34±3.83kPa respectively for PEG, CS and HA. (Figure 2.4)

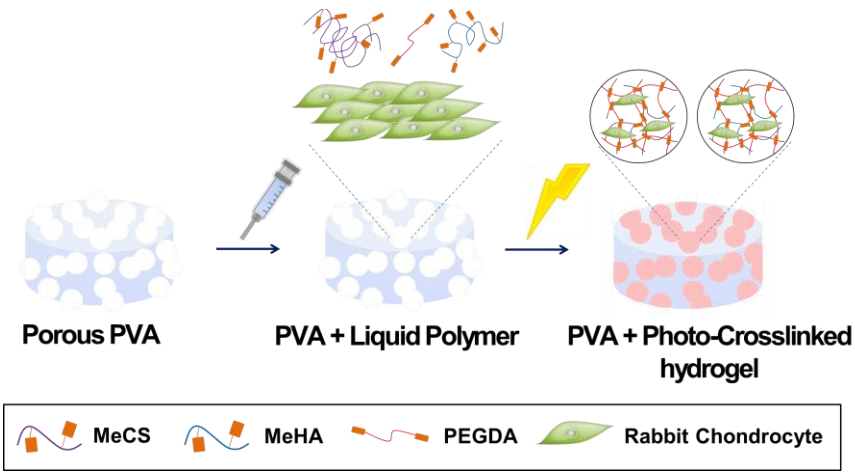

Figure 2.1 Schematic representation of photo-crosslinking ECM-based hydrogels injected into previously prepared porous PVA sponge. Porous PVA was sterilized under UV overnight prior to chondrocytes injection with MeCS and MeHA. Polymer solution homogenously mixed with chondrocytes were added drop-wisely into PVA sponge for UV polymerization. PVA sponge with polymer solution was exposed to UV for photo-polymerization.

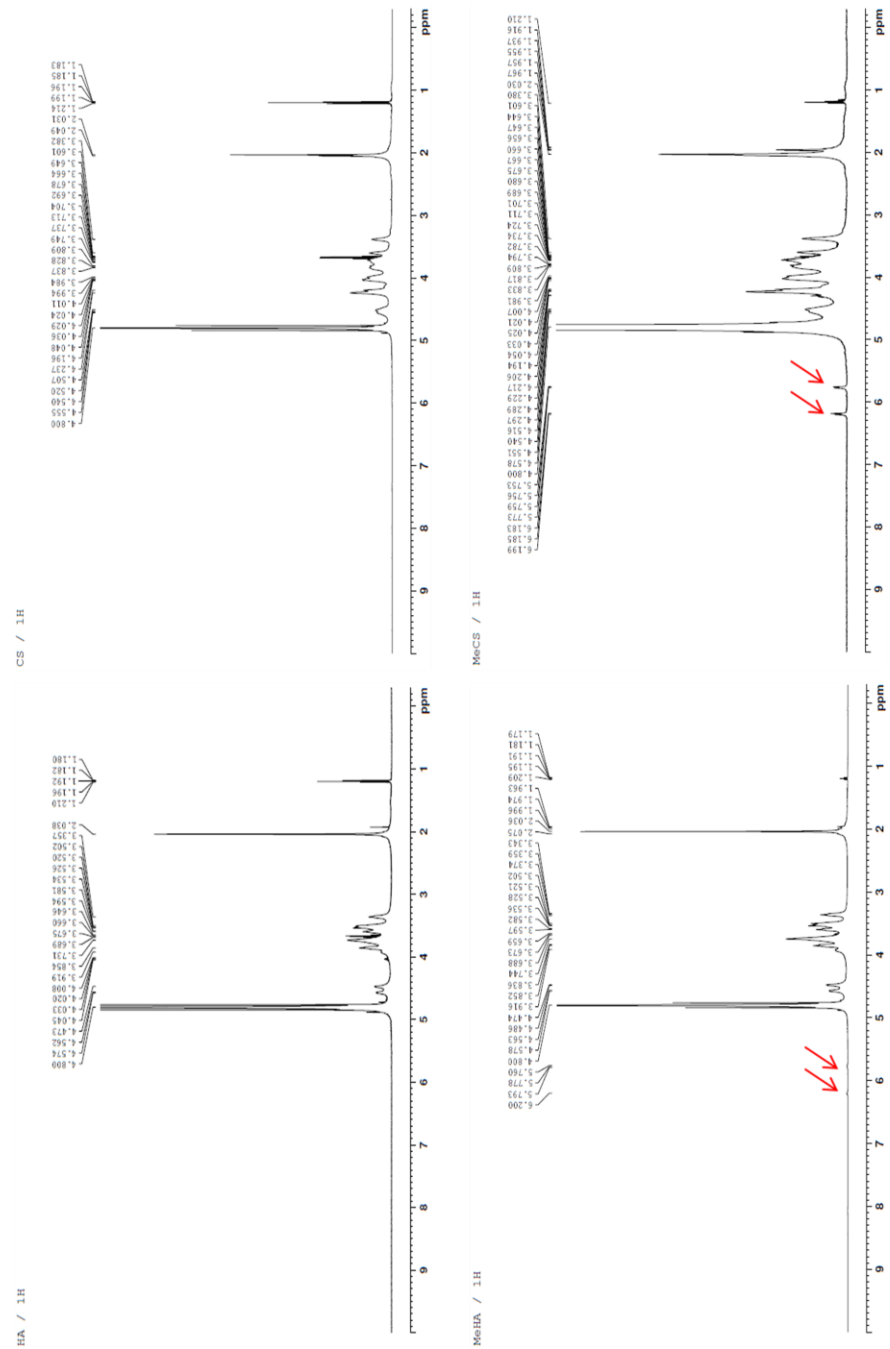

Figure 2.2 ^{1}H-NMR spectra image of CS, HA, MeCS, and MeHA.

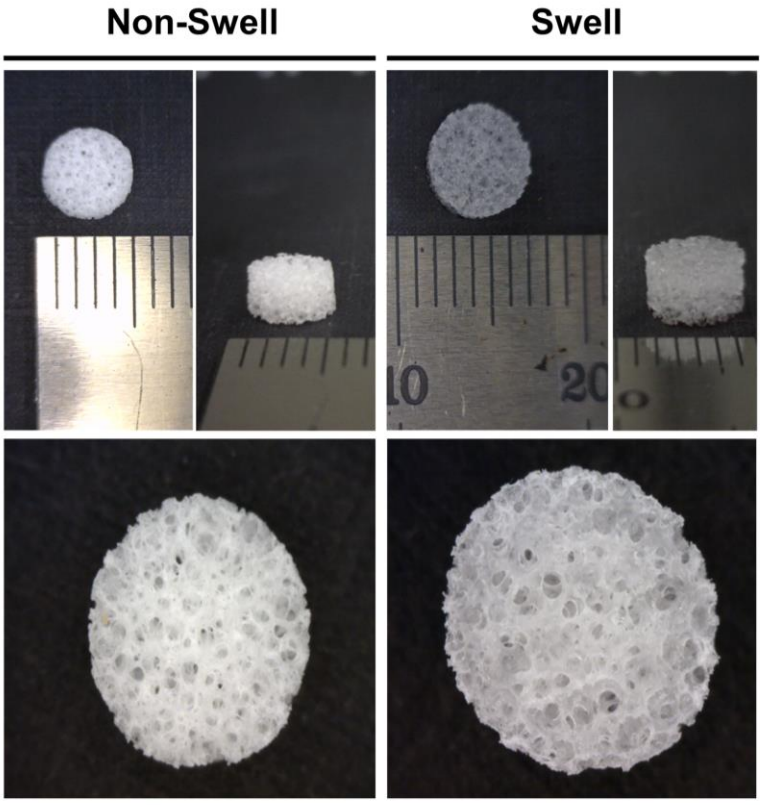

Figure 2.3 Gross images of porous PVA scaffold. Diameter of PVA scaffold is 5mm and height is 2.75mm.

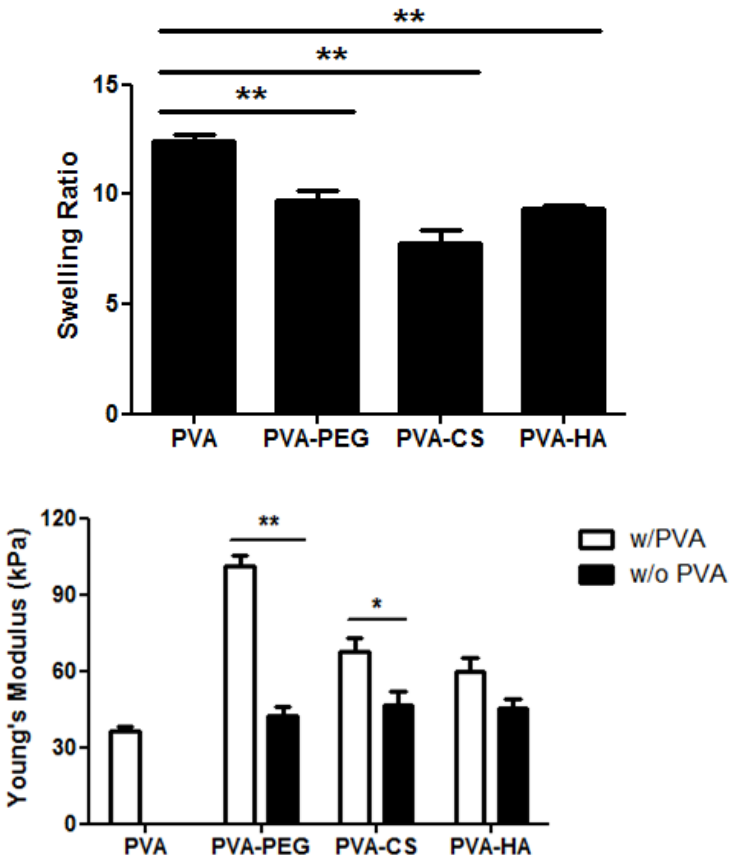

Figure 2.4 (Top) Swelling Ratio of each hydrogel groups after swollen in PBS overnight. PVA indicates PVA only scaffold without photopolymerized hydrogel fusion. (n = 3) (Below) Young's modulus of acellular hydrogels. Without PVA hydrogels are photopolymerized hydrogels only without PVA reinforcement.

2.3.2 Effect of bioactive ECM components on chondrocyte viability

For the cellular study of ECM-based PVA hydrogel, viability of rabbit chondrocytes was examined. Live/Dead assay was performed with samples after 48 hours of seeding or encapsulation of chondrocytes. Rabbit chondrocytes were seeded on the PVA sponge without encapsulation to examine the cellular adhesion on PVA sponge. (Figure 3.5) Images obtained from Live/Dead assay indicated that the chondrocytes rarely adhere to PVA sponge. With the seeding density of chondrocytes as 1×10^6 cells/construct, PVA sponge showed very few cells attached. As a result, further cellular studies have all utilized the chondrocytes-encapsulated ECM-based PVA hydrogels. PVA hydrogel without ECM macromolecules photopolymerized was used as a control. The viability of chondrocytes in ECM-based PVA hydrogels showed above 90% in all three groups. All scaffold composition did not show severe cytotoxicity over two days of cultivation. Viability of chondrocytes in all scaffolds was not significantly affected by photoinitiator used for photopolymerization. (Figure 2.6)

Seeding efficiency of the samples was quantitatively evaluated after 24 hours of cultivation. (Figure 2.6) With the same amount of

chondrocytes seeded on each samples, DNA content measurement indicated significant difference. PVA sponge without photo-encapsulation of cells showed 22.59% of seeding efficiency while other three groups with photo-encapsulation (PVA-PEG, PVA-CS, PVA-HA) showed 97.13%, 97.26%, and 97.67% seeding efficiency, respectively. Chondrocytes hardly attached to PVA sponge while most of the encapsulated chondrocytes were cultured in photopolymerized PVA hydrogels.

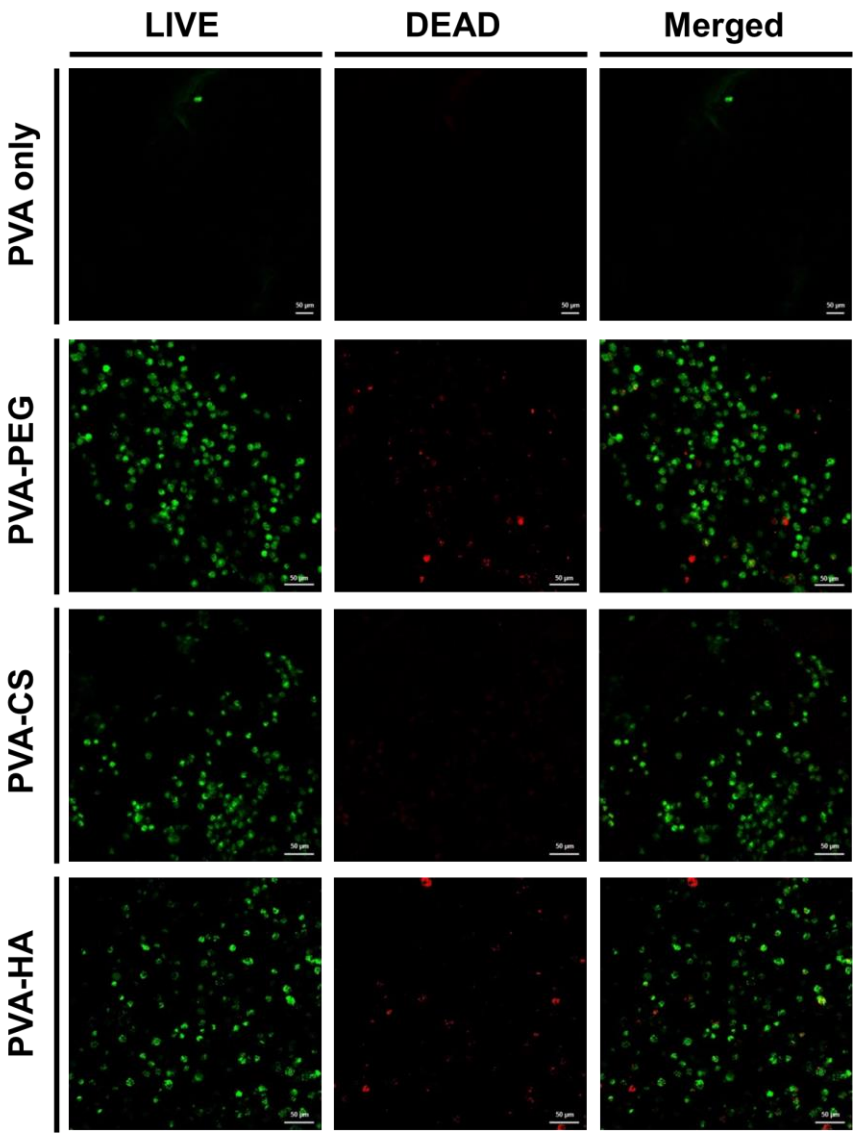

Figure 2.5 Cell viability analysis of encapsulated rabbit chondrocytes via Live/Dead viability Cytotoxicity kit. Live cells stained by green fluorescence (Calcein AM) and dead cells by red fluorescence (Ethd – 1). Scale bar = 50 μm.

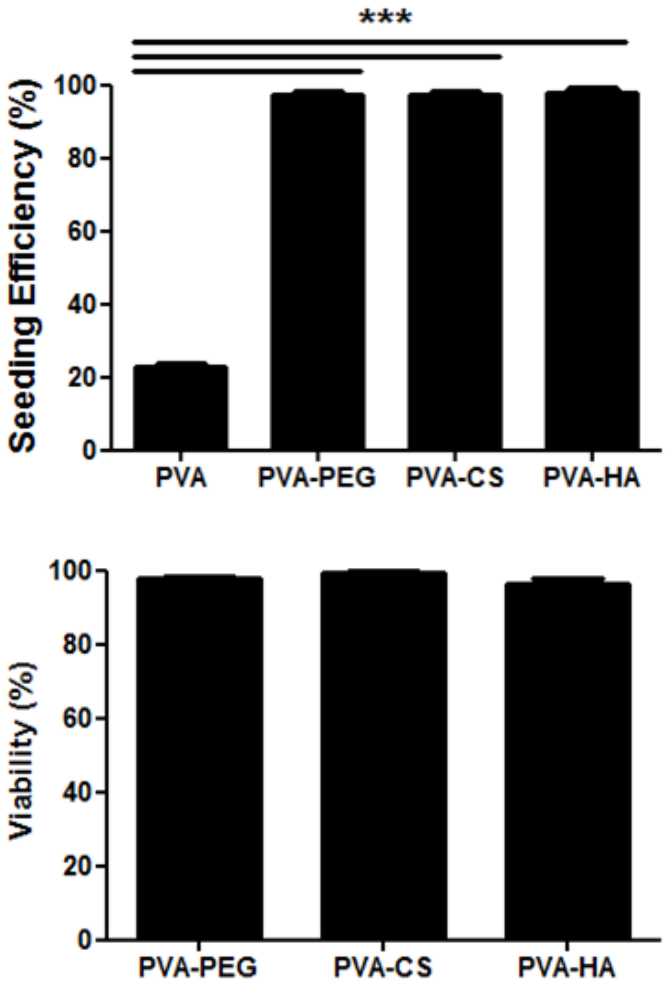

Figure 2.6 (Top) Seeding efficiency of each hydrogel examined via DNA content measurement. (Below) Viability was calculated with the ratio of live cell and total cell number.

2.4 Discussion

PVA is currently being utilized in numerous medical applications, including cartilage tissue repair [15-17]. PVA sponge utilized in this study is highly porous that pores appear apparent with its open cell structure. Macroporous PVA sponge showed large pores with smaller interconnecting pores also observed. (Figure 2.3)

Higher swelling ratio represents hydrogels can retain more water inside it [32]. Swelling ratio of PVA sponge resulted in the decrease with the photopolymerziation. The significant decrease in swelling ration of photopolymerized PVA hydrogel is because the macropores and interconnecting pores where the water is retained are filled with the cross-linked polymer and this prevents the water maintenance inside the pores. The degree of crosslinking may also affect the swelling ratio. The degree of methacrylation of CS and HA that were incorporated into PVA sponge via photopolymerization will affect the crosslinking density which will ultimately influence swelling ratio of hydrogels. (Figure 2.4)

Regarding enhancement of the mechanical strength of the PVA hydrogels, it is mainly contributed to the interpenetrating polymer network through the highly porous PVA sponge. By constructing chemically cross-linked network inside the macroporous PVA

sponge, significant improvement of mechanical property has been revealed for mechanically durable cellular scaffold. (Figure 2.4 Below) This was corroborated by comparing Young's modulus of hydrogels with PVA and without PVA. Compatible with the results of swelling ratio, crosslinking density may also affect Young's moduli of hydrogels. Higher crosslinking density will lead to higher mechanical strength, and vice versa [33]. Therefore, the difference of the Young's moduli among PVA-PEG, PVA-CS and PVA-HA is speculated from the varying crosslinking density. (Figure 2.4)

Due to the highly hydrophilic surface property of PVA, chondrocytes showed very low adhesion to the scaffold [34]. Live/Dead viability assay images of encapsulated rabbit chondrocytes expressed much more live cells stained by green fluorescence than dead cells stained by red fluorescence. (Figure 2.5) This result demonstrates high cell viability and low cytotoxicity of the scaffold over 2 days of cultivation. Accordingly, photopolymerization with photoinitiator did not have critical cytotoxic effect to cell survival. Cellular viability of ECM-based hydrogels did not significantly differ from hydrogels without those components. The presence of bioactive components such as CS and HA did not affect viability considerably. (Figure 2.5 and 2.6) However, significant difference in seeding

efficiency demonstrates rare attachment or adhesion of chondrocytes to hydrophilic PVA surface while effective encapsulation via photopolymerization. (Figure 2.6)

Chapter THREE: Cellular Response of ECM-based PVA hydrogel for Cartilage tissue engineering Application

3.1 Introduction

Articular cartilage has very distinctive composition depending on the depth of the tissue [7]. Depending on the composition and depth of the tissues, cartilage is divided into superficial, middle and deep zone. Superficial zone is made up of the flattened chondrocytes and secreted superficial zone protein (SZP). Middle zone is mainly composited of type II collagen and proteoglycans. The deepest zone has a mineralized matrix adjacent to subchondral bone termed as 'tidemark' [35-37]. These three distinct zonal arrangements closely link to different functions and properties. The superficial zone possesses more tensile and compressive strength that is advantageous to shear resistance from articulating joint surface [23]. Middle zone has aligned collagen fibers connecting upper and deep zone while the deepest zone indicated perpendicular collagen fibers originated from calcified cartilage and larger and more rounded chondrocytes than upper zones [23, 38].

In this chapter, we mainly discuss cellular response of ECM-based PVA hydrogel, which has been proved to display reinforced

mechanical strength compare to conventional photo-crosslinked hydrogels, to clarify the effect of CS and HA incorporation in PVA hydrogel.

3.2 Materials and methods

3.2.1 *Chemicals and reagents*

All chemicals and reagents are purchased from the vendors as mentioned and stored following the manufacturer's instruction: PVA sponge (Medtronic Xomed), Poly (ethylene glycol) diacrylate (PEGDA; MW 3400; Alfa Aesar), Sodium hyaluronate (HA; Lifecore Co.), Chondroitin sulfate (CS; MW 20,000 ~ 40,000; Tokyo Chemical Industry), Glycidyl methacrylate (GMA; Sigma-Aldrich), Photoinitiator (Irgacure 2959), Phosphate buffered saline (PBS; Gibco), Fetal bovine serum (FBS; Gibco), Dulbecco's modified Eagle's medium (DMEM; Gibco), Penicillin/Streptomycin (Pen-Strep; Gibco), 4-(2-hydroxyethyl)-1-piperazaineethanesulfonic acid (HEPES; Gibco), Non-essential amino acid (NEAA; Gibco), L-ascorbic acid (Sigma-Aldrich), L-Proline (Sigma-Aldrich), Deuterium oxide (Sigma-Aldrich), Live/dead Cell viability/cytotoxicity kit (Molecular Probes, L-3224), Quanti-iT^{TM} PicoGreen dsDNA Assay Kit (Invitrogen), Papainase solution

(Worthington Biomedical), Mayer's hematoxylin (Dako), Safranin-O (Polysciences Inc), Eosin Y (Sigma-Aldrich), and M-MLV cDNA synthesis kit (Enzynomics).

3.2.2 *Biochemical analysis*

Biochemical assay was performed on chondrocyte encapsulated PVA hydrogels. Samples were collected after 3 weeks culture and lyophilized for 24 hours. Then the hydrogels were digested with 1ml of papainase solution for 16 hours at 60°C and mechanically crushed with pestle. DNA content was quantified using Quanti-iT^{TM} PicoGreen dsDNA Assay Kit. GAG content was quantified using dimethylmethylene blue (DMMB) spectrophotometric assay at A_{525}, as previously described [39]. Total collagen content was also determined by measuring the amount of hydroxyproline within the samples after acid hydrolysis at 100°C for 18 hours and reaction with p-dimethylaminobenzaldehyde and chloramine-T, as previously described [40]. DNA, GAG and total collagen content was all calculated according to the standard curve. DNA content was normalized by dry weight of the sample and the DNA content of the sample normalized GAG and total collagen content.

3.2.3 *Real-time PCR*

Samples were collected after 3 weeks for real-time PCR. Total RNAs were extracted from the samples with Trizol (Life Technologies). Samples were then physically crushed with the pestle. Using M-MLV cDNA synthesis kit according to manufacturer's instruction, RNA was reverse transcribed into cDNA. Real time – PCR reaction was performed using SYBR Green PCR Mastermix and ABI Step One Plus^{TM} Real time – PCR system. cDNA was amplified by the rabbit primers of the genes in interest such as type II collagen, aggrecan (AGG) , proteoglycan 4 (PRG4), link protein and hyaluronan synthase 2 (HAS2). GAPDH was used as a reference gene. The expression level of genes was calculated as $-2^{\Delta\Delta Ct}$ as previously described [41]. The PCR primers are listed in Table 1.1.

3.2.4 *In vivo transplantation*

For *in vivo* analysis, BALB/c-nude mice at 4 weeks of age (16 ~ 20g body weight, Orient Bio) were anesthetized for subcutaneous implantation of the hydrogels. Rabbit chondrocyte encapsulated hydrogels were cultured *in vitro* in chondrocyte medium for 3 days prior to implantation. Subcutaneously implanted samples were harvested 6 weeks post implantation.

3.2.5 *Histological analysis*

Both *in vitro* (3 weeks) and *in vivo* (6 weeks) samples were fixed with 4% paraformaldehyde at 4°C overnight. Fixed samples were dehydrated in a serial concentration of ethanol followed by xylene. Dehydrated samples were then embedded in paraffin. Paraffin blocks were sectioned into 5μm thick slices and mounted on glass slides. For H&E staining, sections were stained with hematoxyline for 10 minutes, and counter-stained with Eosin – Y for 1 minute. For Safranin-O staining, the sections were stained with Safranin-O for 8 minutes. All sectioned slides were mounted in Canada balsam (Sigma-Aldrich) for further storage and imaging was taken with CKX-41 microscope (Olympus).

3.2.6 *Statistical analysis*

All data are presented as mean ± standard error of the mean (SEM). Statistical significance between groups were determined by Student's t-test with $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

3.3 Results

3.3.1 Effect of bioactive ECM components on chondrocyte proliferation and ECM-based matrix synthesis

Chondrocyte proliferation and ECM-based matrix synthesis were evaluated in perspective of bioactive ECM components addition. (Figure 3.1) Biochemical assay in this study included DNA, GAG and total collagen content measurement. All the samples were collected after 3 weeks *in vitro* culture (n=3). DNA content was quantified by Pico Green assay. DNA content that also indicates cell proliferation showed PVA-CS hydrogel supporting the cell to proliferate most effectively. Additionally, DNA content was statistically significant in PVA-CS hydrogel compared to both PVA-PEG and PVA-HA hydrogel. DNA content was normalized by the dry weight of each sample. Similarly, GAG accumulation was measured by DMMB. GAG accumulation was enhanced in both PVA-CS and PVA-HA hydrogel. Especially PVA-CS showed the most elevated GAG content with significance. GAG amount of PVA-CS and PVA-HA was 16.638 μg/ng and 15.11 μg/ng, respectively. This amount is particularly higher than the amount from PVA-PEG, which was 9.925 μg/ng. Overall, incorporation of ECM components (CS and HA) to the hydrogel benefited GAG

accumulation for better cartilage repair. We also measured the total collagen content. Collagen content was indirectly measured by the calculation of hydroxyproline content. Quantitative analysis of collagen content accumulated by rabbit chondrocyte was significantly higher in PVA-CS, consistent with the result of GAG accumulation. PVA-HA also showed enhanced amount of collagen accumulation compared to PVA-PEG. There was no significance between PVA-CS and PVA-HA for the collagen content. Overall, both CS and HA positively affected encapsulated rabbit chondrocytes on GAG and collagen synthesis with the prominent result from PVA-CS. Exogenous supplement of CS to the chondrocytes encapsulated PVA hydrogels was confirmed to show the most promising result in ECM accumulation. All the values of GAG and collagen amount were normalized to the values of DNA content. (Figure 3.1)

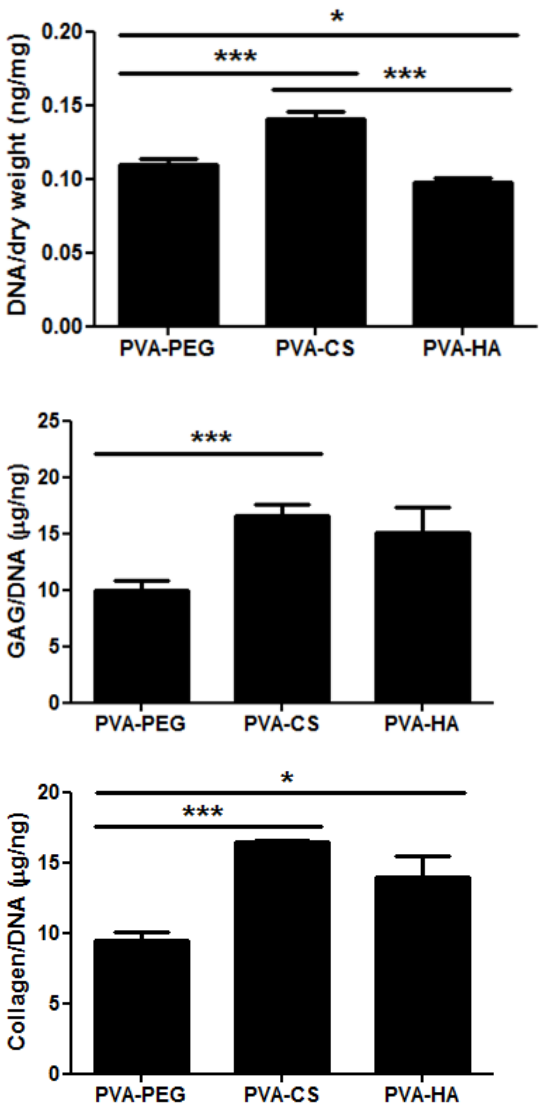

Figure 3.1 Quantification of cell proliferation (DNA) and matrix accumulation (sGAG and total Collagen) of chondrocytes cultured *in vitro* were evaluated by biochemical assays.

3.3.2 Gene expression analysis of chondrocytes in PVA hydrogels

Furthermore, in order to examine how effective bioactive ECM component on the cartilage regeneration, we investigated gene expression via real-time PCR for the 3 week-cultured hydrogels: PVA-PEG, PVA-CS and PVA-HA (n=3). Gene expression we were interested in was cartilage related genes, specifically cartilage ECM accumulation related genes; type II collagen, aggrecan, proteoglycan 4 (PRG4), link protein and hyaluronan synthase 2 (HAS2). (Figure 3.2) GAPDH was used as a reference gene. All gene expression was normalized to PVA-PEG control for the same culture period. The results showed the gene expression level relatively differ according to hydrogel composition. To be specific, Type II collagen and aggrecan was up-regulated in PVA-CS. Expression level of type II collagen was more elevated in PVA-CS comparing to both PVA-PEG and PVA-HA. This result corresponds with GAG and total collagen content measurement. Type II collgen and aggrecan showed 0.99-fold and 0.83-fold respectively in PVA-HA. On the other hand, proteoglycan 4 (PRG4) known as lubricin is highly over-expressed in PVA-HA compared to other groups. It appeared to have 15-fold in PVA-HA while PVA-CS indicated 0.5-fold. PRG4 gene is generally known to encode superficial zone protein (SZP) used as synonym of

lubricin [42]. Lubricin, having highly related structure with superficial zone protein, has a significant role in lubrication of articular joints and minimizes the coefficient of friction of the cartilage surface [43-45]. Similar expression was observed in link protein. Link protein, also one of the components of cartilage extracellular matrix, acts as a stabilizer of the aggregates of aggrecan and hyaluronic acid [46]. Incorporation of MeHA resulted in up-regulated expression of link protein, significantly. Relative fold induction indicated 1.5-fold in PVA-HA. Hyaluronan synthase 2, hyaluronic acid synthesizing enzyme, also showed up-regulation for both PVA-CS and PVA-HA. However, slightly higher fold was observed in PVA-HA (1.4-fold) than PVA-CS (1.3-fold), even though there was no significance of the expression level between all three groups. From the above results, incorporation of CS enhanced GAG synthesis of chondrocytes, while HA showed the greatest response in terms of lubricin synthesis and proteoglycan deposition. (Figure 3.2)

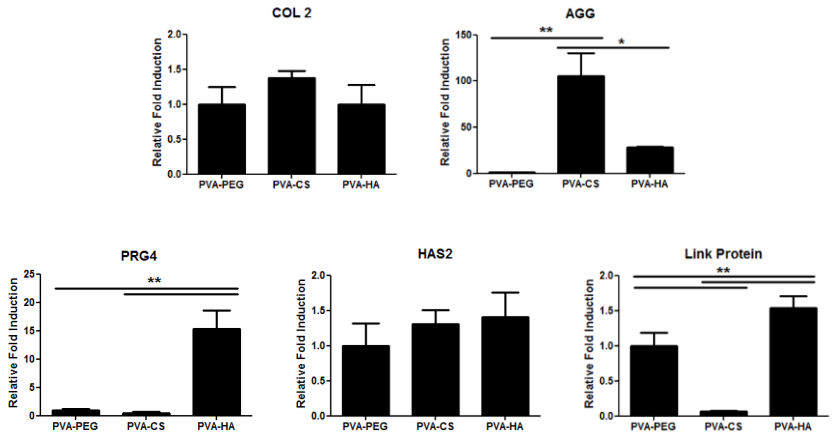

Figure 3.2 Relative gene expression level for Collagen II, Aggrecan, Proteoglycan 4 (PRG4), Hyaluronan Synthase 2 (HAS2) and Link Protein after 3 weeks culture. Gene expression was normalized to PVA-PEG for the same time period culture and GAPDH was used for the reference housekeeping gene.

3.3.3 Compressive modulus of hydrogel cultured *in vivo* and *in vitro*

Cellular hydrogels were prepared for mechanical test. All the test condition was same as acellular hydrogel compression test. PVA hydrogels were cultured in chondrocyte medium for 3 weeks *in vitro* whereas *in vivo* subcutaneous implantations were retained for 6 weeks.

Interestingly, Young's modulus of PVA-CS culture *in vitro* significantly increased compared to acellular hydrogel. PVA-PEG and PVA-HA did not show indicative increase or decrease in modulus after 3 weeks *in vitro* culture. Among three different groups of particular hydrogel composition, PVA-CS had the highest mechanical stability presented by the highest Young's modulus. Apparent from stress-strain curve, we observed the steepest slope in linear region of PVA-CS that represents the highest Young's modulus. (Figure 3.3)

In vivo samples subcutaneously implanted for 6 weeks showed slightly distinctive results. PVA-PEG and PVA-CS had comparable modulus to *in vivo* samples, but still higher modulus than acellular hydrogels. On the other hand, PVA-HA obtained remarkably increased Young's modulus in comparison with both acellular and *in vitro* samples. Therefore, influence of HA was outstanding for *in*

vivo response. Stress-strain curve clearly illustrate the highest slope in PVA-HA. (Figure 3.4)

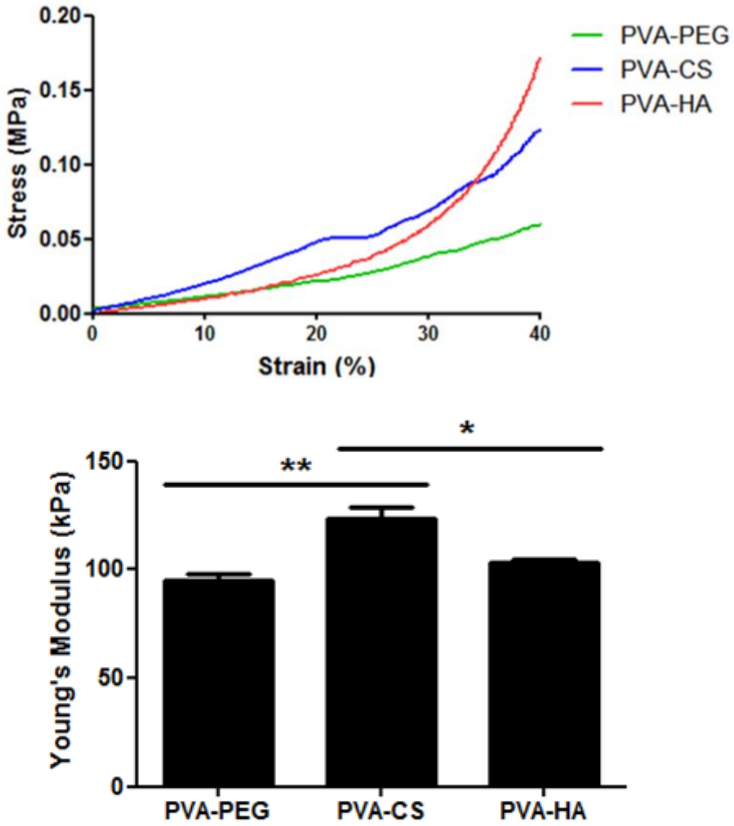

Figure 3.3 (Top) Stress-Strain curve of *in vitro* 3 weeks culture hydrogels. Stress was applied until the 40% of original hydrogel height at 1.5mm/min rate. (Below) Young's Modulus of cellular hydrogel after 3 weeks *in vitro* culture. (n = 3)

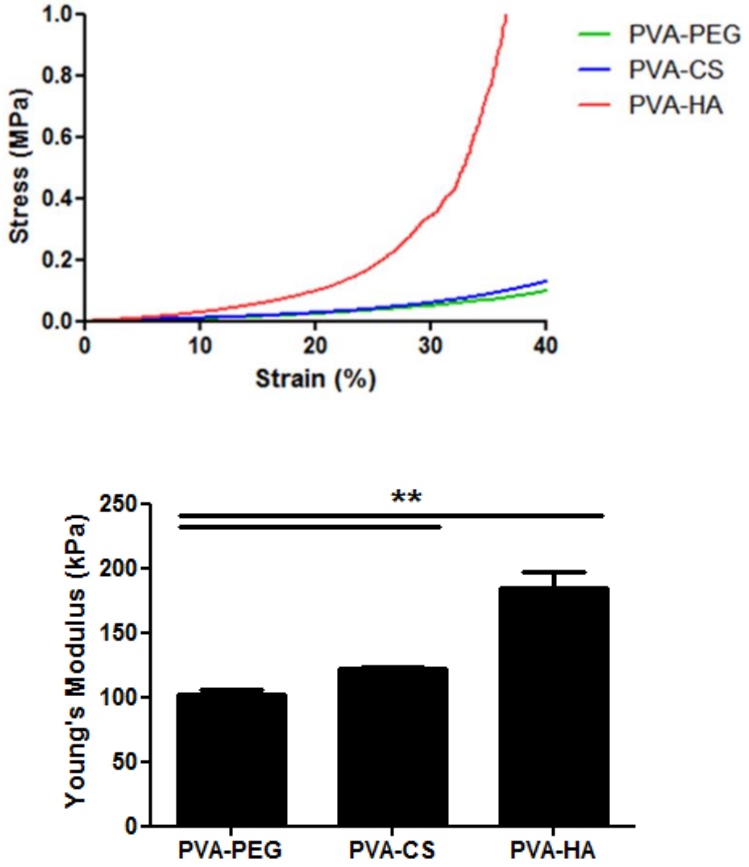

Figure 3.4 (Top) Stress-Strain curve of *in vivo* 6 weeks subcutaneously implanted hydrogels. (Below) Young's Modulus of cellular hydrogel after 6 weeks *in vivo* implantation. (n = 3)

3.3.4 Histological analysis of *in vitro* and *in vivo* engineered cartilage tissue

Histological evaluation of chondrocytes cultured for 3 weeks *in vitro* and 6 weeks *in vivo* were performed with H&E and Safranin-O staining. Cross section of each hydrogels was stained to assess the ECM synthesis. Both H&E and Safranin-O staining obviously presented porous PVA structure and photo-crosslinked polymers immersed in PVA.

For hydrogels cultured *in vitro* for 3 weeks, H&E staining exhibited cell encapsulation in photopolymerized polymers rather than attached to PVA. Chondrocytes were more evenly distributed throughout photopolymerized hydrogels in PVA-CS and PVA-HA than PVA-PEG. Therefore, cell proliferation is more retained in PVA-CS and PVA-HA than PVA-PEG. From Safranin-O staining, we observed more intense and vivid red staining in PVA-CS. Although PVA-CS contains CS itself in hydrogel, still PVA-CS demonstrates positive staining of accumulated sulfated glycosaminoglycans (sGAG). This result is consistent with biochemical and compressive analysis that presented better chondrocytes' response in PVA-CS. (Figure 3.5)

To examine the effect of CS and HA under *in vivo* circumstance, subcutaneously implanted samples were collected and histologically

stained. In comparison to *in vitro* evaluation, we noticed that more dense and compact tissue was formed. Numerous cells were visualized with H&E staining. Safranin-O staining featured great and intensive staining in PVA-CS and PVA-HA. Both PVA-CS and PVA-HA resulted in matrix synthesis particularly sGAG without severe inflammatory response. (Figure 3.6)

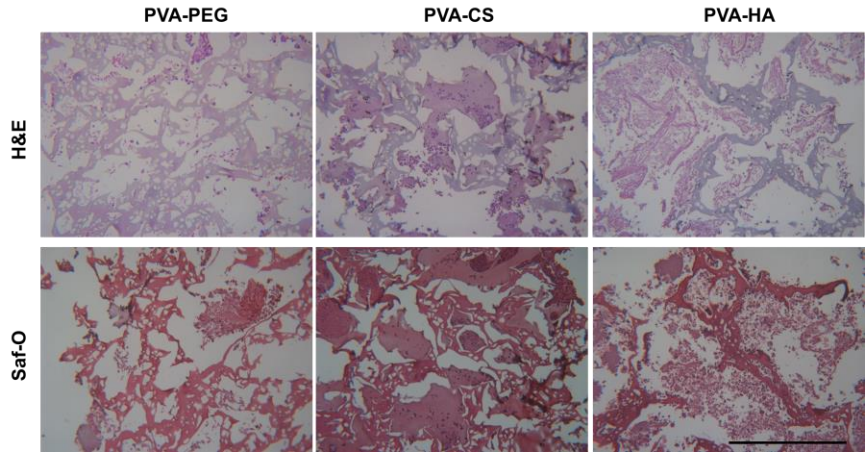

Figure 3.5 H&E and Safranin-O staining of *in vitro* 3 weeks culture hydrogels. Scale bar = 100 μm.

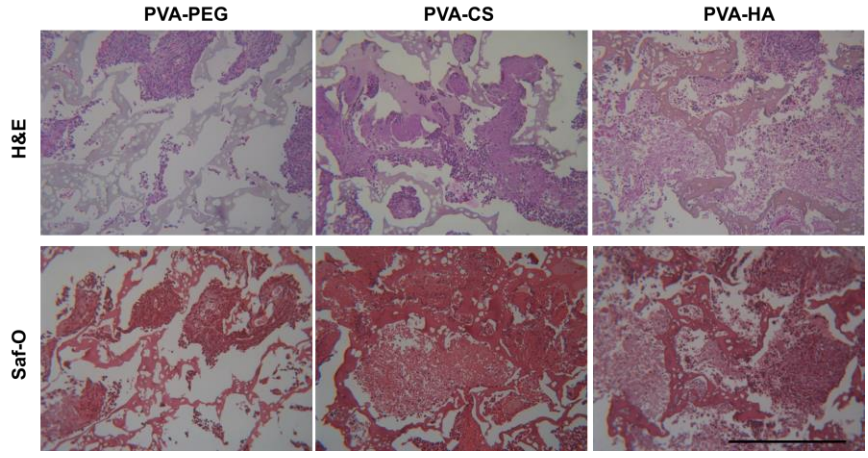

Figure 3.6 H&E and Safranin-O staining of *in vivo* 6 weeks culture hydrogels. Scale bar = 100 μm.

3.4 Discussion

Cellular responses of encapsulated rabbit chondrocytes showed various results according to hydrogel composition. Cell proliferation and accumulation of ECM macromolecules such as sulfated GAG and collagen increased in both CS and HA incorporated hydrogel with slight better response with CS supplement. (Figure 3.1) This result is consistent with a gene expression level specifically type II collagen and aggrecan. Up-regulation of these genes refers to the promotion of anabolic activity of chondrocytes for better cartilage repair by increase in cartilage major structural components [24]. Elevated expression of PRG4 in PVA-HA indicates that by tailoring microenvironment of chondrocytes, boundary lubrication can be promoted remarkably for functional cartilage regeneration. (Figure 3.2) Previous studies proposed that chondroitin sulfate based 3D scaffold activated cartilage specific gene markers, specifically type II collagen and aggrecan [47, 48]. Moreover, there was a report about interactions between aggrecan and collagen. This report suggests that chondroitin sulfate rich region of aggrecan interacts with the collagen fibrils of cartilage and this interaction improves mechanical properties [49]. With the respect to articular cartilage structural components like type II collagen and aggrecan, CS appeared to be an

effective bioactive component for cartilage tissue engineering. However, in terms of articular cartilage functional components like PRG4, link protein, and HAS2, HA was observed to be better agent than CS for cartilage regeneration. PRG4, link protein and HAS2 are all related proteins for joint physiology. PRG4 (lubricin) itself functions as a lubricant to protect the gliding surface of articular cartilage and prevent degradation of the joint [50, 51]. Link protein and HAS2 are both particularly engaged in hyaluronic acid stabilization [46] and synthesis [52]. Hyaluronic acid (HA), backbone of the aggrecan, provides the optimal viscosity level for the outflow minimization during joint motion addition to the interaction with lubricin to lower the viscosity of hyaluronan solution [50]. Therefore, the role of link protein and HAS2 are also important for cartilage lubrication. Consequently, enhanced expression level of PRG4, link protein and HAS2 with HA incorporation claims that HA is a necessary component for functional cartilage repair. This study has identified that chondroitin sulfate and hyaluronic acid in cooperation with chondrocytes displayed different effect on the cartilage related gene expression. (Figure 3.2)

Compressive modulus measurement also displayed different result depending on the components of hydrogel. Hydrogels cultured *in*

vitro for 3 weeks period resulted in higher Young's modulus with CS incorporation. This result has strong correlation with *in vitro* biochemical assay analysis that proved the effect of CS on chondrocytes' behavior in ECM reconstruction. However, subcutaneously implanted hydrogels *in vivo* cultured for 6 weeks showed higher Young's modulus with HA incorporation. Despite different chondrocytes' response *in vitro* and *in vivo*, all samples resulted in reinforced mechanical property compared to acellular hydrogels without PVA sponge. (Figure 3.3 and 3.4)

Furthermore, histological staining of *in vitro* samples exhibited strong and vivid staining of both H&E and Safranin-O in PVA hydrogel with CS (PVA-CS) that suggests better cell proliferation and sGAG synthesis of chondrocytes. Both PVA-CS and PVA-HA expressed compact and dense tissue cultured *in vivo*. As a result, CS and HA supplement into PVA-PEG signified substantial enhancement of cellular response for cartilage tissue engineering. (Figure 3.5 and 3.6)

Conclusion

In this study, we focused on fabrication of PVA reinforced ECM-based photopolymerizing hydrogel for enhanced mechanical properties and cellular responses. Cartilage-specific ECM deposition was increased in PVA-CS and PVA-HA. Cartilage-related mRNA expression levels were distinctive according to hydrogel composition (CS or HA). Subcutaneous implantation of ECM-based hydrogels reinforced with porous PVA led to a condensed tissue formation via encapsulated chondrocytes' ECM molecule synthesis. Overall, our study demonstrates mechanically durable PVA incorporated with ECM-based hydrogel for biological enhancement, specifically promising lubricin in response to CS.

Table 1.1

Gene	Sequence (5' -3')
GAPDH	F: TCA CCA TCT TCC AGG AGC GA R: CAC AAT GCC GAA GTG GTC GT
Collage Type II	F: TTC ATG AAG ATG ACC GAC GA R: GAC ACG GAG TAG CAC CAT CG
Aggrecan	F: CCT TGG AGG TCG TGG TGA AAG G R: AGG TGA ACT TCT CTG GCG ACG T
Proteoglycan 4 (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
Hyaluronan Synthase 2 (HAS2)	F: TTT CTT TAT GTG ACT CAT CTG TCT CAC CGG R: ATT GTT GGC TAC CAG TTT ATC CAA ACG G

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국문초록

연골 조직 재생을 위한 다공성 PVA와 세포외기질 기반의 하이드로겔 합성

관절 연골은 혈관이 존재하지 않는 결합조직으로 매우 제한적인 자가 재생능력을 가지고 있다. 그러나, 연골의 손상은 연골이 신체의 하중을 버티는 능력과 관절 움직임의 윤활 작용을 감소시키는 기능 장애를 유발시키는 가장 큰 원인 중 하나로 알려져 있다. 폴리비닐알코올 (PVA) 하이드로겔은 비분해성과 생체적합성을 가진 FDA 승인 합성 생체재료로 알려져 있다. 본 연구에서는 다공성 PVA 스펀지에 생리활성 물질을 채운 하이드로겔을 제작하여 연골 재생에 활용하고자 하였다. 광중합성 황산 콘트로이틴 (Chondroitin Sulfate, CS)과 하이알루론산 (Hyaluronic Acid, HA)을 토끼 연골세포와 혼합하여 다공성 PVA 스펀지에 주사함으로써 하이드로겔을 제작하였다. 연구는 CS가 첨가된 PVA 하이드로겔에서 세포외기질의 합성과 연골 관련 유전자 발현이 증가 됨을 확인할 수 있었다. 또한, HA가 첨가된 PVA

하이드로겔에서는 연골의 윤활 작용에 관여하는 단백질의 유전자 발현이 증가하는 것으로 나타났다. 이러한 결과들을 토대로, 본 연구는 PVA와 생리활성 물질의 융합을 이용한 견고한 하이드로겔이 더 나아가 기능적인 연골 조직공학에 이용될 수 있음을 입증 할 수 있었다.

주요어: PVA (Poly Vinyl Alcohol), Hydrogel, Hyaluronic Acid, Chondroitin Sulfate, Chondrocyte, Cartilage Tissue Engineering

학번: *2014-20571*