공학석사 학위논문

Fabrication of photo-crosslinked and RGD modified ECM-based hydrogels for functional cartilage tissue engineering

연골 조직 공학을 위한 아르지닌-글리신-아스파르트산 펩타이드와 세포외기질 기반의 광중합성 하이드로겔 합성

2014 년 6 월

서울대학교 대학원

공과대학 화학생물공학부

김 환

Fabrication of photo-crosslinked and RGD modified ECM-based hydrogels for functional cartilage tissue engineering

연골 조직 공학을 위한 아르지닌-글리신-아스파르트산 펩타이드와 세포외기질 기반의 광중합성 하이드로겔 합성

지도 교수 황 석 연

이 논문을 공학석사 학위논문으로 제출함

2014 년 6 월

서울대학교 대학원

화학생물공학부

김 환

김 환의 석사 학위논문을 인준함

2014 년 8 월

위 원 장 김 병 기 (인)

부위원장 황 석 연 (인)

위 원 백 승 렬 (인)

Abstract

Fabrication of photo-crosslinked and RGD modified ECM-based hydrogels for functional cartilage tissue engineering

Hwan Drew Kim
School of Chemical and Biological Engineering
The Graduate School
Seoul National University

Articular cartilage damage is a persistent and increasing problem with an aging population. Though it is not life threatening, damages do strongly affect one's quality of life. Such damages can be resulted from a variety of causes, such as traumatic accident or wear and tear over time. Articular cartilage does not easily go through the regeneration step after having injuries or disease leading to loss of tissue and formation of a defect. Therefore, strategies to achieve complete cartilage restoration remain a challenge. Photopolymerizing-based injectable hydrogels have long received attention in the cartilage tissue engineering, due to their unique biocompatibility, flexible method of synthesis, range of constituents, and desirable physical characteristics. In the present study, we have introduced unique bioactivity within the photopolymerizing hydrogels by copolymerizing polyethylene glycol diacrylate (PEGDA) macromers with various photo-crosslinkable extracellular matrix (ECM) molecules (hyaluronic acid and chondroitin sulfate) and integrin binding peptides (RGD peptide). Results indicate that the cellular morphology as observed by the

actin cytoskeleton structures, was strongly dependent on the types ECM components as well as the presence of integrin binding moieties. Furthermore, chondroitin sulfate (CS)-based hydrogel with integrin binding moieties increased the lubricin (or known as superficial zone protein, SZP) gene expression by the encapsulated chondrocytes. Additionally, CS-based hydrogel with RGD peptide increased DNA, GAG, and collagen contents compared HA-based gel and non-modified control hydrogels. This study demonstrates that integrin-mediated ECM microenvironments should be considered for the hydrogel-based cartilage tissue engineering applications.

Keywords: Hydrogel, Bovine Chondrocyte, SZP, RGD, Chondroitin Sulfate, Hyaluronic Acid, Cartilage Tissue Engineering

***Student Number*: 2012-23263**

TABLE OF CONTENTS

LISF OF FIGURES AND TABLE

Figures

TABLE

CHAPTER ONE: THE SCIENTIFIC BACKGROUND AND RESEARCH PROGRESS

1.1 Overview

One of the goals of tissue engineering is to develop a tissue construct to repair or replace damaged organs. Conventional tissue engineering strategies involves seeding cells into a 3D structure called a scaffold, to which the cells can attach and grow [1, 2]. However, *in vivo* tissues have complex cellular organization and more precise arrangement of cells need to be employed. In order to engineer a complex architecture that is similar to organs *in vivo,* engineered tissues need to be composed of varying cell types. Previously, attempts have been made to create organized tissue by plating multiple cell types on a porous scaffold system prior to *in vivo* transplantation [3, 4]. For this scaffold system, over the past few decades, biomaterials research has made tremendous advancements. Biomaterials can be defined as tissue-contacting materials, which can perform particular biomedical functionality. Biomaterials, such as polymer, ceramics, metal, transplant tissue or composite materials have been extensively used in drug delivery, gene therapy, wound healing, cosmetic formulation, biosensors, and contact lens.

A variety of synthetic polymers, including poly (L-lactic acid) PLLA, poly (glycolic acid) (PGA), poly (ethylene glycol) (PEG), polycaprolactones, polyorthoesters, and polycarbonates have been used to fabricate 3D scaffolds for tissue engineering. These materials are approved from Federal and Drug Administration (FDA), and these

materials can be tailored to meet certain tissue-specific mechanical and degradation properties by controlling the molecular weight and co-polymerize with other components. However, many of the synthetic biomaterials are inert and, therefore, does not provide bioactive signal necessary for stem cell differentiation. Furthermore, synthetic scaffold biomaterials may illicit potentially inflammatory responses caused by acidic degradation products. Despite these disadvantages, these synthetic materials can be tailored to provide controlled degradation behavior in vivo and matched mechanical properties with native tissues. To illicit biological activities, many of these materials can be chemically modified and transform into biologically active scaffolds.

1.2 Polyethylene glycol diacrylate (PEGDA) hydrogel

Hydrogels provide the unique properties to encapsulate various cells, creating cell-laden 3D scaffolds for developing and creating a new tissue. In addition to its capacity to encapsulate cells, hydrogels are materials that have a high-swelling ratio, which allows for efficient and direct transport of external nutrients to the cell, excretion of waste products. Moreover, most importantly, mechanical properties that resemble the viscoelastic properties of native tissue make them potentially useful as tissue engineering scaffolds. Among many types of synthetic or natural polymers, Polyethylene glycol (PEG) based hydrogels were prepared in order to study because suitability for biomedical applications. PEG, with its hydroxyl moieties, can be acrylated to form PEGDA (polyethylene diacrylate). After mixing with photoinitiator, exposure to UV radiation yield radicals. These radicals initiate polymerization, by attacking the C=C double bonds present in the acrylate groups. (Figure 1.1) In linear

chain formation, branched as well as crosslinked structures are also formed, thus forming an insoluble three-dimensional polymer network. After cellular encapsulation, cells retain spherical and circular phenotype in hydrogels. However, PEGDA hydrogels do not provide cell-specific adhesions, and these are also non-degradable. Therefore, incoporation of biological moieties and degradation units should be addressed for successful PEGDA hydrogel based tissue engineering applications. .

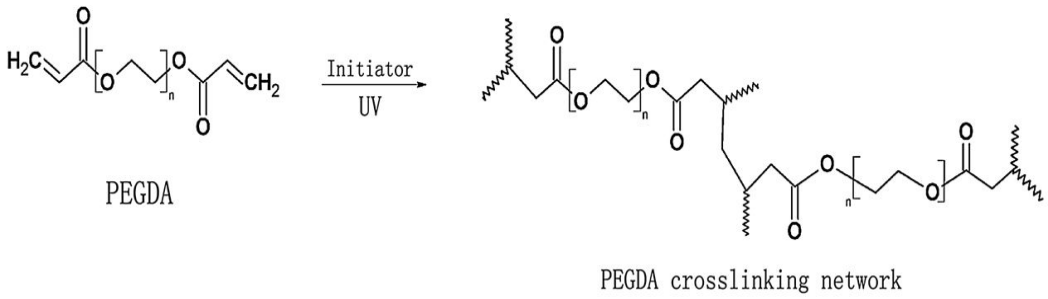

Figure 1.1 Chemical structures of PEGDA and the reaction [5]

1.3 RGD peptide and extracellular matrix (ECM)components

As I mentioned in above, PEGDA hydrogels are poor substrates for cell and protein adhesion because of their lack of cell-specific nature. Adhesion-promoting oligopeptides such as sequence of RGD (arginine-glycine-aspartate), implicated in cellular attachment via integrin, have often been incorporated into the hydrogels to improve their adhesion properties, particularly for adhesion-dependent cells. Anchorage independent cells such as chondrocytes exhibit good cell viability within RGD-modified hydrogels. Moreover, extracellular matrix (ECM) provides structural and biochemical supports that enhance cell to cell communication, mediate

differentiation, and encourage the proliferation of cells. They mostly consist of glycosaminoglycan and proteins. Chondroitin sulfates and hyaluronic acids are most abundant type of glycosaminoglycan in our cartilage tissue that incorporation with PEGDA hydrogel will provide cell to have ECM rich microenvironment.

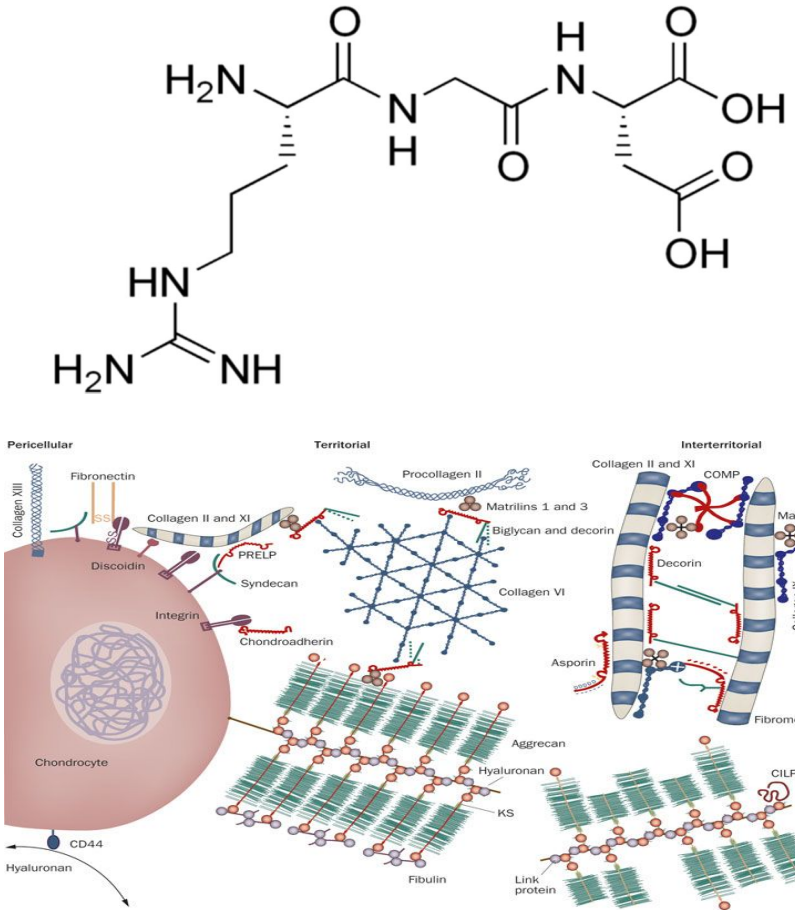

Figure 1.2 Chemical structures of Arginine-Glycine-Aspartate (RGD) peptide and the cartilage matrix surrounding chondrocytes in healthy articular cartilage is arranged into zones defined by their distance from the cell [6]

1.4 Cartilage tissue engineering

Tissue engineering approaches for articular cartilage repair utilizing bioactive scaffolds has recently attracted enormous attention in the field regenerative medicine field [7]. Cartilage tissue engineering strategies seek to overcome the cartilage self-repair limitation through the development of cellular scaffolds that closely mimics the complex structure of articular cartilage. Cartilage is composed of specialized cells called chondrocytes with dense fibrillar network of collagen type II and large proteoglycans containing negatively charged glycosaminoglycan [8]. Furthermore, articular cartilage is organized in a functional manner, in which superficial (or boundary) layer of articular cartilage secrets is lubricating factors such as lubricin (superficial zone proteins) and hyaluronic acids [9]. However, when cartilage damages happened, chondrocytes cannot migrate to the damaged area because of aneural and avascular characteristic of cartilage. Therefore, deposition of matrix is slow in progress. (Figure 1.3)

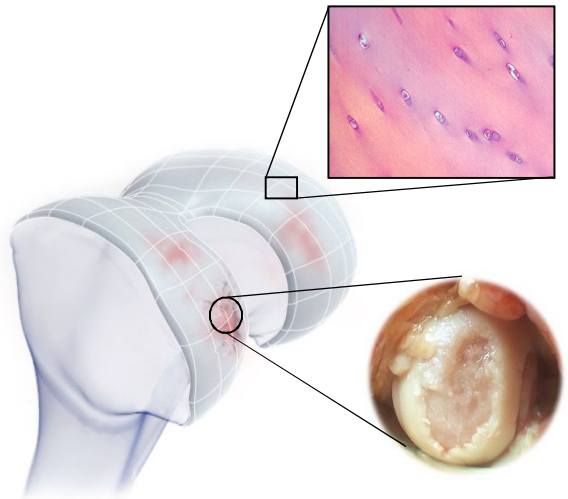

Figure 1.3 Overview of articular cartilage and damaged cartilage

Hydrogels exhibit a biomolecular structure that is similar to native tissue [10]. It develops as an indispensable requisite for chondrocyte transplantation that serve as a foundation template for increasing cell to cell interactions and the formation of extracellular matrix (ECM) to provide structural support to the newly formed tissue. In particular, polyethylene glycol diacrylate (PEGDA)-based photopolymerizing hydrogels have been extensively used as scaffolds for cartilage tissue engineering applications primarily because of the ease of chondrocyte encapsulation, ease of handling [11] and create the particular functional group to the hydrogel. [12] Furthermore, PEGDA-based system has been utilized for cartilage tissue engineering utilizing mesenchymal stem cells [13, 14], bone tissue engineering utilizing mesenchymal stem cells [14] and embryonic stem cells [15, 16]. However, PEG-based system exhibits poor biological properties attributable to their bio-inertness [17, 18]. To overcome these limitations, PEG-based systems with mixed ECMs have been developed to introduce bioactivities in PEG-based hydrogels [19]. In addition, methacrylated biological macromolecules have been photo-crosslinked along with PEG-based system to provide appropriate biological properties for stem cell differentiation [20].

1.5 Research aims

Recently, we have reported that chondrocytes isolated different zones (superficial/middle/deep) seeded within ECM-based hydrogels showed varying cellular response [21]. Therefore, to mimic innate cellular microenvironment, improved ECM

compositions are necessary to ensure the long-term maintenance of the chondrocyte characteristics. The present study tests the hypothesis that the incorporation of RGD into ECM-based hydrogels may serve as a biologically active microenvironment that supports chondrocyte phenotype. ECM biomolecules such as chondroitin sulfate and hyaluronic acids were chemically modified to have methacrylate groups, and these ECM biomolecules were utilized to form hydrogels with RGD sequence or RDG sequences and compared with PEG-based hydrogels. We examined whether cell-integrin interactions are modulated by the cellular microenvironments by examining viability, production of cartilage ECM materials, and gene expressions. Furthermore, cellular morphology, as observed by the actin cytoskeleton structures, was strongly dependent on the types ECM components as well as the presence of integrin binding moieties. In addition to support of cellular phenotype, the expression of lubricin and hyaluronic acid synthase (HAS) are a substantial challenge for articular cartilage tissue engineering. In this regards, we show that the expression of lubricin and HAS gene expressions were modulated by ECM-specific microenvironments and integrin interactions. This study provides a framework for understanding the interactions between chondrocytes and the ECM components and integrin-mediated cellular functions.

CHAPTER TWO: THE SYNTHESIS OF CELL RESPONSIVE ECM-BASED HYRDROGELS

2.1 Introduction

The methacrylate-conjugation of chondroitin sulfate (CS) and hyaluronic acids (HA) with glycidyl methacrylate (GMA) is a novel strategy for introducing photocrosslinkable unit to polysaccharides that can generate ECM-based hydrogels by UV light-induced polymerization. Since CS and HA are one of the major components of native articular cartilage, responsible for the tissue's compressive strength and also capable of absorbing large volume of water, CS and HA should be considered as optimal materials for cartilage. Moreover, the tripeptide Arg-Gly-Asp (RGD) mediates both cell-substratum and cell-cell interactions. Therefore, we hypothesized that CS and HA based hydrogels with RGD incorporation may be idealistic biomaterials for cartilage tissue engineering.

2.2 Materials and methods

2.2.1 Isolation and culture of bovine chondrocytes

Bovine Chondrocytes were isolated, cultured, and characterized as previously described [22]. Briefly, full thickness bovine cartilage was obtained from the slaughterhouse. The collected cartilage samples were processed within 24 hours. Cartilage pieces were incubated in Dulbecco's modified eagle's medium (DMEM, Gibco) containing 5% fetal

bovine serum (FBS, Gibco) and 0.2% collagenase (Worthington Biochemical Corporation, USA) for 14-18 hours. Isolated chondrocytes were then filtered with 70 ㎛ mesh and washed with phosphate-buffered saline (PBS). Cells were maintained with DMEM containing 10% FBS, 10000 U/ml penicillin-streptomycin, 1 mM of *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid* (HEPES; Gibco), 100 μM of Non-Essential Amino Acid (NEAA, Gibco), 0.4 μm of proline, and 50 μg/ml of vitamin C. Culture medium was changed every other day.

2.2.2 Methacrylation of ECM components (Chondroitin Sulfate and Hyaluronic Acid)

Chondroitin sulfate (CS; Sigma-Aldrich) and hyaluronic acid (HA; MW 1,600,000 Genzyme Corp) all have polysaccharide structure with hydroxyl functional group (-OH). Hydroxyl functional group was reacted with the glycidyl methacrylate (GMA; Sigma-Aldrich) in order to form a crosslinkable acrylate. The reaction was conducted as previously described [23]. For methacrylated HA (MeHA), the same protocol was followed as methacrylated CS (MeCS). For MeCS, 1.0 grams of CS dissolved in PBS (10%) were reacted with 1 ml of GMA for 11 days. For MeHA, 1.0 grams of HA dissolved in PBS (1%) were reacted with 2 ml of GMA for 8 days. After the reaction, methacrylated CS and HA were in dialysis for 48 hours and replenished with fresh water 4 times against 1000 MW dialysis membrane. Then they were lyophilized and stored at -20℃ until use.

2.2.3 Photoencapsulation

Collected P0 bovine chondrocytes were encapsulated into tyrosine-arginine-glycine-aspartate-serine (YRGDS)-modified poly (ethylene glycol)-diacrylate (PEGDA, SunBio Inc., Korea) or tyrosine-arginine-glycine-serine-aspartate (YRGDS)-modified PEGDA, as previously described [15]. To produce RGD modified polymer, YRGDS were synthesized using solid phase peptide synthesis (SPPS). Briefly, YRGDS and YRDGS were reacted with acrylate-PEG-*N*-hydroxysuccinimide-ester (acrylate-PEG-NHS, 3500 MW; Jenkem Technology in 50 mM TRIS buffer (pH 8.2) for 2 hours at room temperature. HPLC was performed to confirm RGD and RDG peptide purity (Figure. 2.1). Mass spectroscopy was confirmed the YRGDS molecular weight of 595.05 (Figure. 2.2) The product was lyophilized and the final concentration of the polymer solution (20% w/v) containing 2.5 mM RGD and RDG was prepared by mixing PEGDA and Acrylate-PEG-YRGDS or Acrylate-PEG-YRDGS in sterile PBS. RGD/RDG modified PEGDA solution was mixed with MeCS (10% w/v in PBS) and MeHA (1% w/v in PBS), respectively, in 1:1 ratio to formulate pre-gel polymer solution. Cells were gently mixed with a polymer solution at a concentration of 2×10^6 cells/ml and photopolymerized with photoinitiator (Igacure 2959, 0.05% w/v) using UV light (3.5 mW/cm^2) for 5 minutes. The constructs (total volume of 75 µl) were then cultured at 37°C with 5% CO_2 in 2.5 ml chondrocyte culture medium for 3 weeks. In addition, for in situ cell staining, thin film hydrogels with 500 µm were fabricated using glass template.

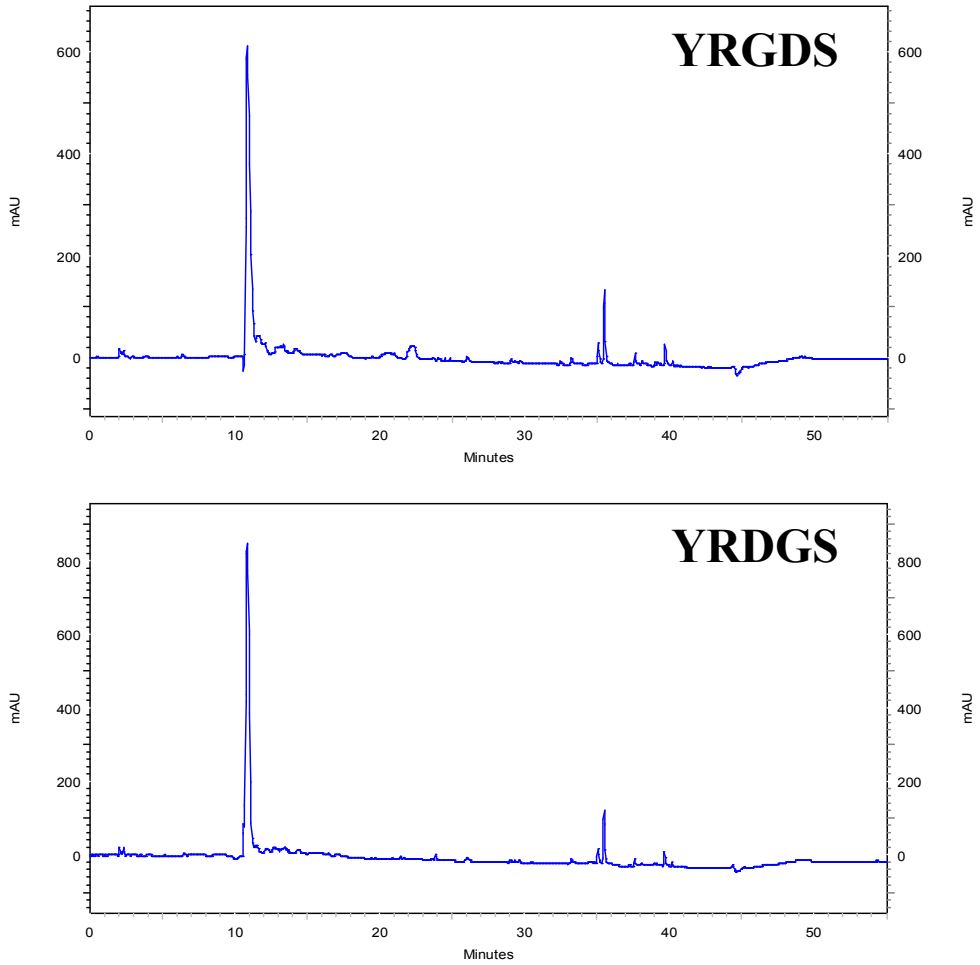

Figure 2.1 HPLC chromatogram of synthesized of YRGDS. Upper channel: UV detection at 230nm. Lower channel: UV detection at 260nm. YRGDS is observed at the retention time of 10-11min. YRDGS. Upper channel: UV detection at 230nm Lower channel: UV detection at 260nm. YRDGS is observed at the retention time of 10-11min.

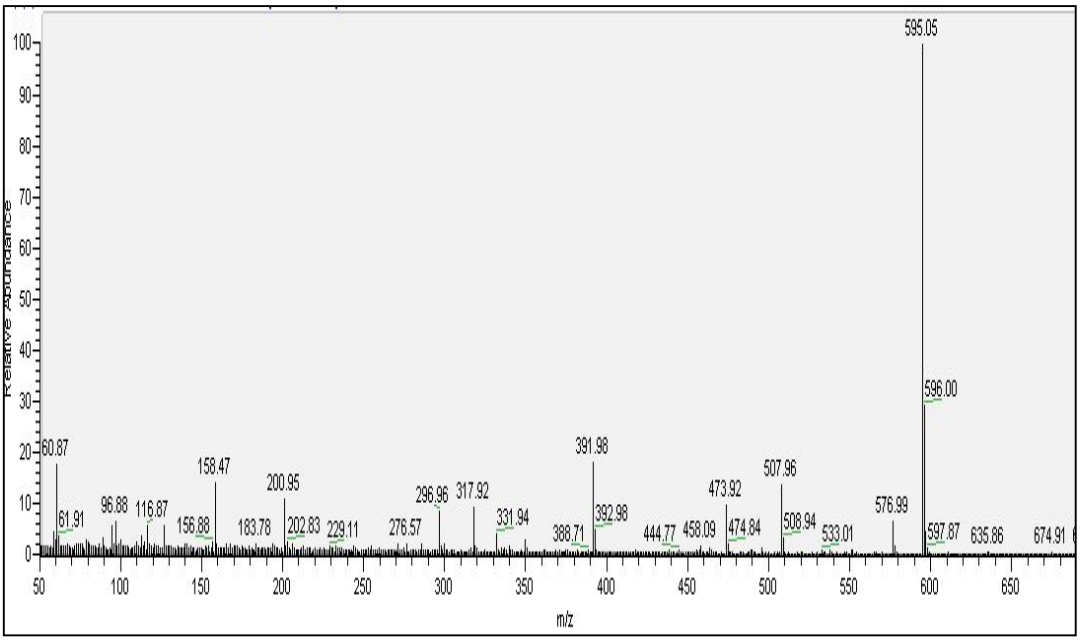

Figure 2.2 Mass Spectroscopy of synthesized of YRGDS. Molecular weight of the major product is 595.05 Da

2.2.4 Swelling properties and mechanical testing

The swelling ratios of the hydrogels were calculated from the following relation as described previously. Swelling ratio = Weight of the equilibrated gel / Weight of the dry gel. Hydrogels were swollen in PBS for a day before doing the compression test, and Instron Model 5966 (Instron Corporation, MA, USA) was used for measuring Young's modulus of each hydrogel. The pressure applied to the surface of the hydrogel and the distance between hydrogel and compressor were used to calculate stress and strain. Young's modulus was obtained by stress-strain curve. Degradation of modified hydrogels was also examined with respect to weight loss. Weight loss of initially weighed hydrogels (W_0) were monitored as a function of incubation time in PBS with Hyaluronidase (100 unit/ml, H3506, Sigma-Aldrich) and Chondroitinase (1 unit/ml, C3667, Sigma-Aldrich) at 37°C. At specified time intervals (1 to 4 days), hydrogels were removed from the PBS and weighed (W_t). The weight loss ratio was defined as $100\ \% \times (W_0 - W_t)/W_0$.

2.2.5 Statistical analysis

All data are expressed as mean$\pm$standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA single factor) with * $p < 0.0$

2.3 Results

2.3.1 Design and synthesis of photo-crosslinkable biomacromolecules for RGD/RDG-modified ECM-based hydrogels

Figure 2.3 shows brief scheme of methacrylation of CS and HA that have functional hydroxyl group that can react with GMA, resulting in addition of photo-reactive vinyl groups. CS and HA dissolved in PBS were reacted with GMA for 11 and 8 days, respectively. Methacrylated CS (MeCS) and HA (MeHA) were dialyzed against 1000 MW membrane, lyophilized, and NMR was performed and confirmed the existence of acrylate functional group on CS and HA backbone (Figure. 2.4). Two NMR peaks of Me CS representing vinyl protons at 6.207 ppm and 5.768 ppm and MeHA of 6.193 ppm and 5.771 ppm were observed in the NMR spectra. RGD peptide was incorporated into monoacrylated PEG chain by the reaction of $–NH_2$ group of RGD or RDG with –NHS group of ACRL-PEG-NHS. Then MeCS and MeHA were mixed with ACRL-PEG-RGD/RDG 1:1 ratio to make RGD modified ECM-based hydrogel construct. Overall scheme of synthesis of photo-crosslinkable and RGD modified ECM-based hydrogels shows in Figure 2.5. RGD-modified ECM-based hydrogels were crosslinked with Irgacure2959 photoinitator using UV light. Gross images of each type of acellular hydrogel revealed regular and uniform (Figure. 2.6).

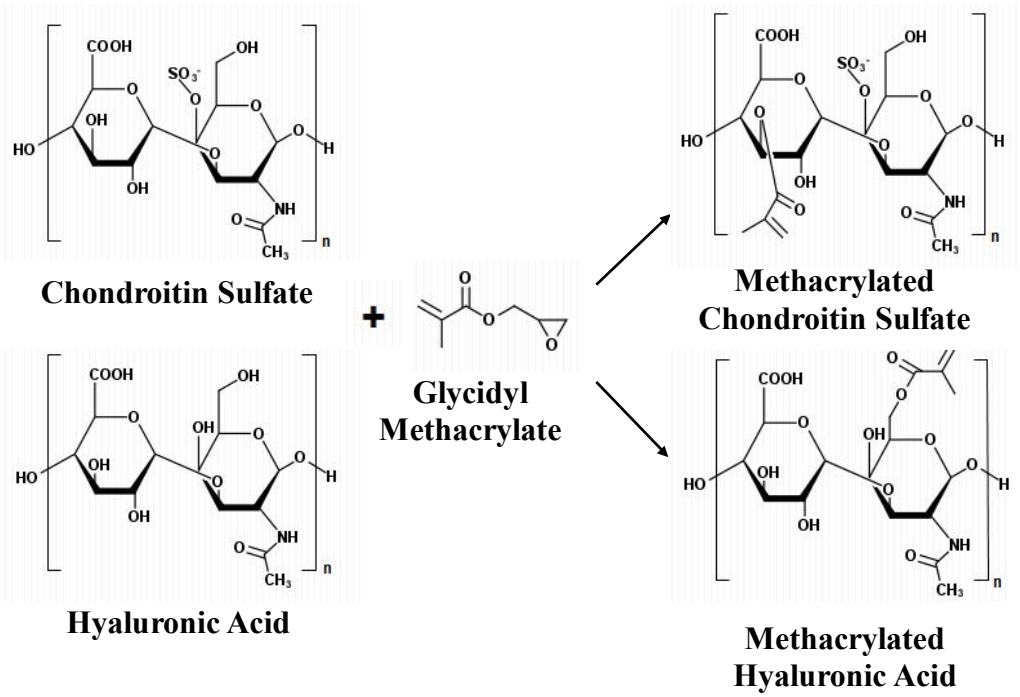

Figure 2.3 Schematic illustration of the transesterification reaction. Methacrylation of Chondroitin Sulfate (CS) and Hyaluronic Acid (HA) that they were reacted with Glycidyl Methacrylate (GMA) to form Methacrylated CS and Methacrylated HA.

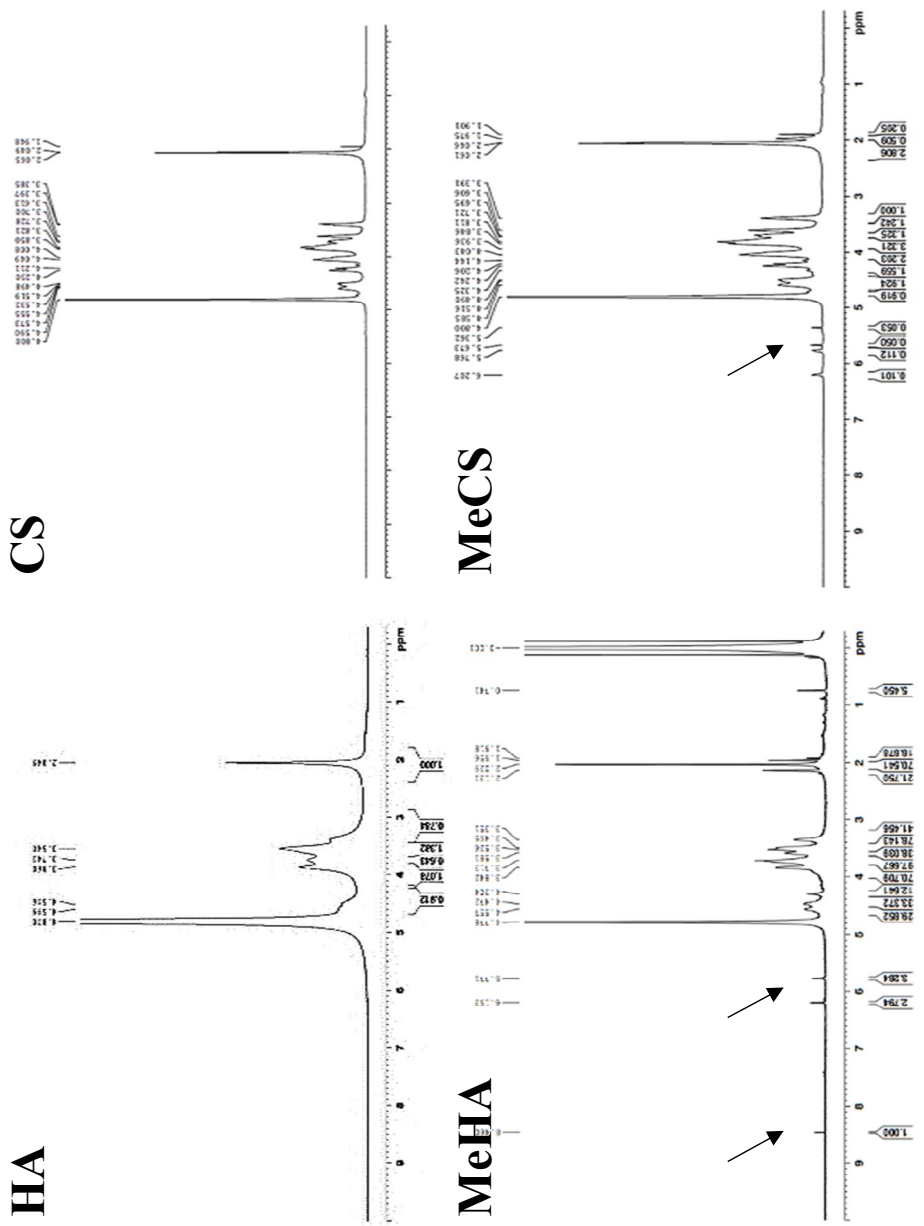

Figure 2.4 ^{1}H-NMR spectra image of CS and MeCS. Acrylate peaks at ~5.3ppm to ~6.2ppm indicate the presence of MeCS and ^{1}H-NMR spectra image of HA and MeHA. Acrylate peaks at ~ 5.7ppm to ~ 6.2ppm indicate the presence of MeHA.

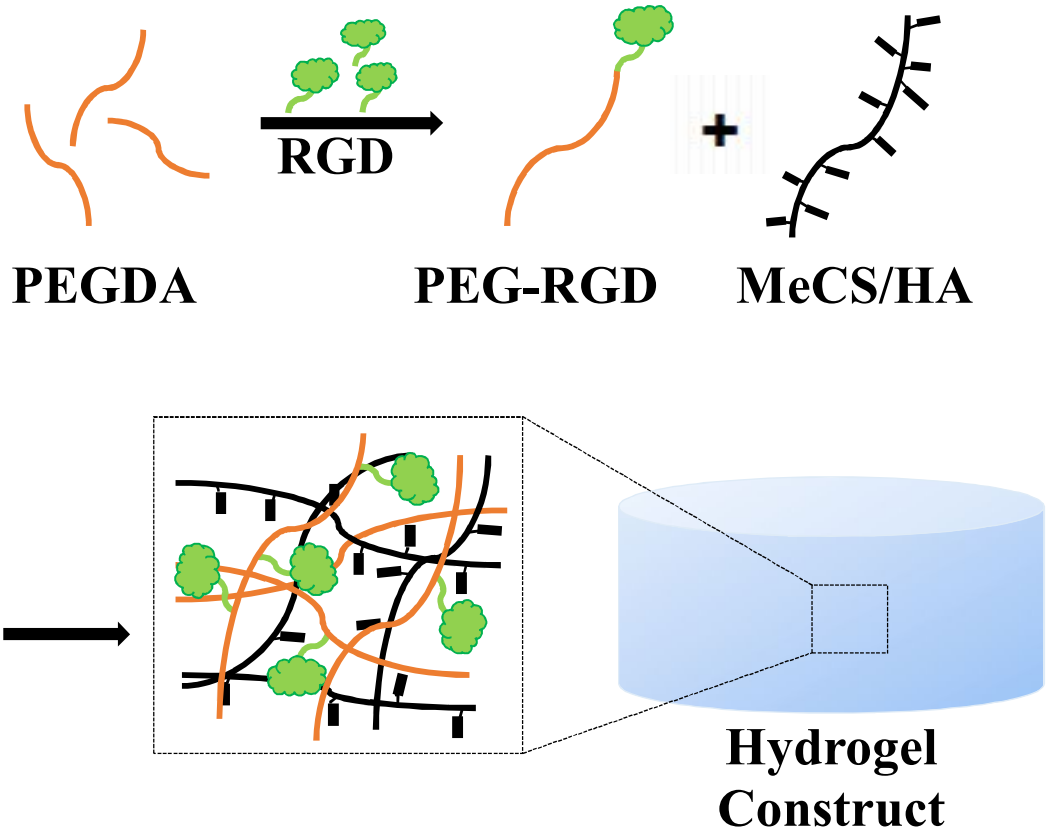

Figure 2.5 ACRL-PEG-RGD/RDG was mixed with MeCS and MeHA respectively to make a different group of hydrogel

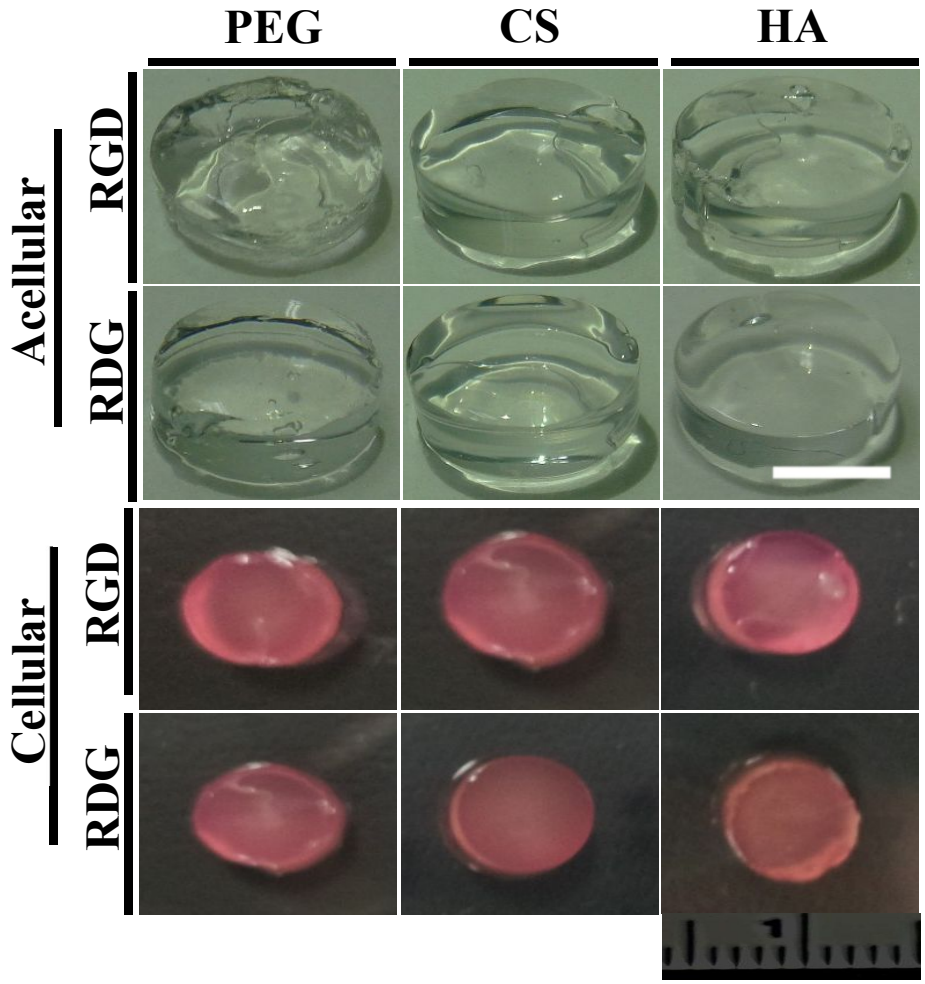

Figure 2.6 Gross image of the acellular and cellular hydrogels. Scale bar = 250 μm.

2.3.2 Characterization of RGD/RDG-modified ECM-based hydrogels

Acelluar hydrogels were characterized for their swelling behavior, Young's modulus and degradation. The equilibrium swelling ratio of RGD/RDG-modified ECM-based hydrogels is shown in Figure 2.5. Chondroitin sulfate (CS) hydrogel group had a swelling ratio of 14.2 which was higher than that of HA-hydrogel (13.1). Negatively charged sulfate groups within CS molecule may have caused an increase in water content, thus increasing the gel compressive modulus (Figure. 2.2) [24]. Incorporation of RGD/RDG groups into the ECM-based hydrogel did not cause any change in swelling ratio.

For degradation study, weight losses of initially weighed hydrogels were monitored as a function of incubation time in PBS with hyaluronidase and chondroitinase at 37°C. Both acellular HA-RGD and HA-RDG hydrogels have similar degradation properties (12.91 % and 13.61 % of mass reduction, respectively) after 4 days of incubation under hyaluronidase. For acellular CS-RGD and RDG hydrogels, their degradation properties were also comparable that 28.12 % and 27.54 % of mass was reduced by chondroitinase after 4 days. Because of different amount of ECM concentration inside of hydrogel (1% of HA and 10% of CS) made some difference in mass reduction. (Figure. 2.6).

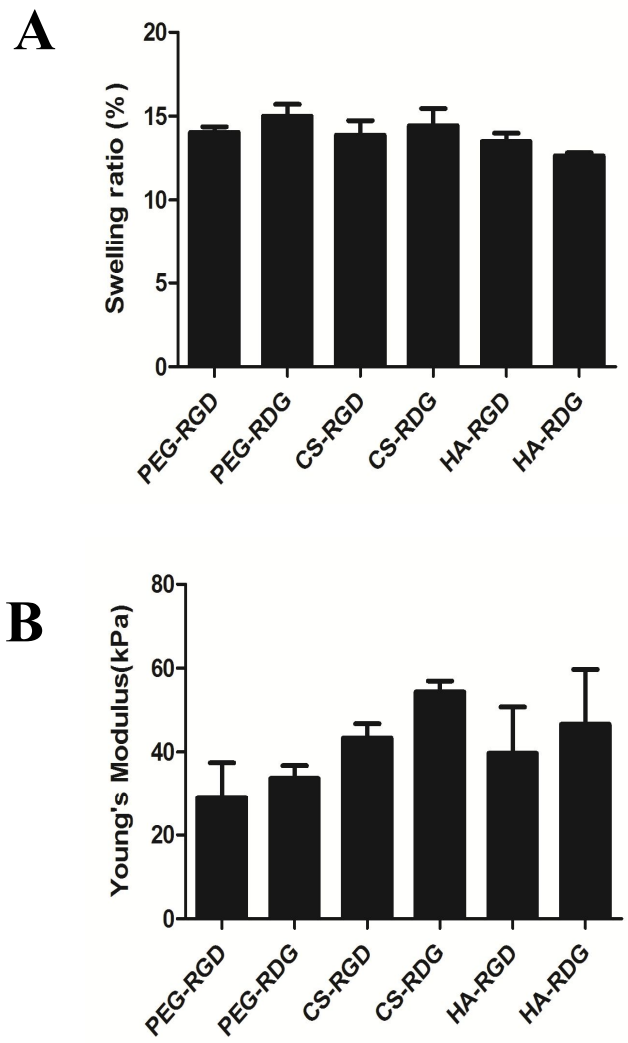

Figure 2.7 RGD/RDG-modified ECM-based hydrogels characterization. (A) Swelling ratio of 6 hydrogel group after swollen in PBS for 1day. Similar swelling ratio was observed between hydrogel groups. Error bars represent the standard deviation on the mean for n=3. (B) Young's Modulus values for acellular 6 hydrogel group. Error bars represent the standard deviation on the mean for n=3.

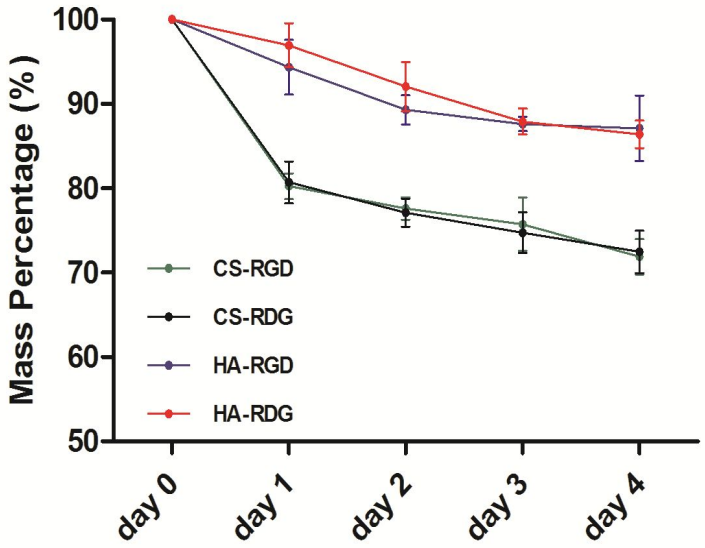

Figure 2.8 Degradation rate of each type of hydrogel materials. Error bars represent the standard deviation of the mean for n=3. Viability test within the hydrogel after cultured for 24hr.

2.4 Discussion

Photopolymerization has been utilized in numerous tissue engineering applications, including cartilage repair [25-27]. PEG-based photopolymerizing hydrogels provide a confined template in which the encapsulated cells function and produce matrix; however, they lack cellular interactions. In order to define whether ECM components can influence stem cell behavior, we have previously investigated the effects of extracellular matrix (ECM) components in three-dimensional (3D) microenvironment by creating PEG-based hydrogels with exogenous type I collagen, type II collagen, and hyaluronic acids (HA) [28, 29]. These studies strongly suggest that the ECM-dependent modulation of stem cell fate, indicating the importance of ECM microenvironment. In addition to exogenously introducing ECM components to hydrogels, numerous chemical options are available for modifying biological macromolecules to undergo photopolymerization. In particular, polysaccharides have been in receipt of much attention as materials of biomimetic scaffold because their structures are similar with several extracellular matrix (ECM) molecules found in natural tissue. Several studies have utilized chemically modified CS and HA for hydrogel formation and applied them in tissue engineering applications. For example, CS-based hydrogel significantly influenced chondrogenesis of mesenchymal stem cells due to cell clustering during culture time [28]. Also, mesenchymal stem cells encapsulated hyaluronic acid based hydrogel induced the chondrogenesis via CD44 receptor that plays a decisive role in sensing microenvironment and controlling intracellular signal pathway [30]. However, synergistic effects of ECM components and integrin interactions have not been reported for cartilage tissue engineering. In the present study, we have created ECM-based

hydrogel by modifying CS and HA with photo-crosslinkable functional groups. Furthermore, we introduced Arg-Gly-Asp (RGD) peptide into our ECM-based hydrogels system to provide integrin-specific interactions.

CHAPTER THREE: TISSUE ENGINEERING APPLICATION OF CELL RESPONSIVE ECM-BASED HYRDROGELS

3.1 Introduction

A novel cell responsive ECM-based hydrogels were developed through the ECM methacryalation and RGD peptide conjugation into the PEGDA hydrogel scaffold for cartilage tissue engineering. Compared with control hydrogel, which does not have ECM or RGD peptide, chondrocytes show large proliferation activity, and exhibit a stable cell phenotype. Glycosaminoglycans such as chondroitin sulfates and heparin sulfate are major components of the cartilage extracellular matrix. These proteoglycans have been reported to play roles in cell proliferation and differentiation through interactions with cells and cytokines [31]. This chapter plays a role in investigating the effect of chondroitin sulfate and RGD peptide for chondrocytes transplantation in hydrogels.

3.2 Materials and methods

3.2.1 Cellular viability and morphological analysis

Viability of encapsulated cells in hydrogels was determined by Live/Dead Cell viability/cytotoxicity kit (Molecular Probes, L-3224, Eugene, OR) that contains calcein-AM ("Live" dye) and ethidium homodimer01 ("Dead" dye). Images were collected

using CKS41 fluorescence microscope (Olympus, JAPAN). For cellular morphological analysis, cells were fixed at 4% paraformaldehyde and cut into 500 μm sections. After permeabilized with Triton X-100 for 30 min, hydrogel was stained with 4'6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 10 minutes and Alexa Fluor® 488 Phalloidin (A12379, 1:150 dilutions, Life Technologies) for 2 hours. Images were obtained using a confocal microscope (LSM 510-Meta NLO, Zeiss, Germany)

3.2.2 Biochemical analysis

Biochemical assays were performed on RGD/RGD-modified ECM-based hydrogels constructs. Constructs (n = 3) were collected at 3 weeks, lyophilized, digested in papainase solution (1 ml/construct; 125 μg/mL Worthington Biomedical, Lakewood, NJ) for 16 hours at 60°C [22], and mechanically crushed. DNA content was quantified using Quant-iT™ PicoGreen®dsDNA Assay Kit (Invitrogen™, Carlsbad, CA, USA) followed by the manufacturer's instruction. The GAG content was quantified using dimethylmethylene blue (DMMB) spectrophotometric assay at A_{525}, as previously described [32]. For the measurement of proteoglycan produced by cells in PEG-CS hydrogels, we have subtracted off the GAG value of acellular PEG-CS hydrogels from the total value (*i.e.* GAG from CS-PEG + cells). Total collagen content was determined by measuring the hydroxyproline content of the constructs after acid hydrolysis and reaction with *p*-dimethylaminobenzaldehyde and chloramine-T, as previously described [33]

3.2.3 Histological and immunofluorescence analysis

Cell-laden hydrogel constructs were fixed with 4% paraformaldehyde solution at 4°C for overnight. Prior to the embedding in optimal temperature cutting compound (OCT), samples were incubated in 20% sucrose solution for 2 hrs at room temperature, and OCT-embedded samples were placed in isopentane, frozen in liquid nitrogen. Using a cryostat (Leica CM3050), samples were cryosectioned into 15 µm-thick slices. For H&E staining, frozen sections were rehydrated in PBS at room temperature for 10 mins and they were stained with hematoxylin (Ricca Chemical Company, Gill 2, cat#: 3536-16) for 5 mins and thoroughly rinsed with DI water. Sections were then stained with Eosin-Y (Richard-Allan Scientific, cat#: 7111) for 1 min, followed by multiple washes in DI water. For Safranin-O staining, the rehydrated sections were stained with 0.1% Safranin-O (ScholAR Chemistry, NY) for 5 mins at room temperature and then samples were gradually dehydrated in the series of ethanol, followed by CitriSolv (Fisher scientific, cat#: 22-143975). For immunofluorescence staining of collagen type I, II, and F-actin, rehydrated sections were blocked and permeabilized in PBS containing 0.3% Triton X-100 and 3% bovine serum albumin (BSA, Sigma, cat# A7906) for 1 hr at room temperature. Next, sections were incubated with primary antibodies against type I and type II collagens (1:200; rabbit polyclonal, Fitzgerald, cat# 70R-CR007X and cat# 70R-CR008X, respectively) at 4°C for overnight. After washing in PBS, sections were incubated with secondary antibodies (1:250; goat anti-rabbit Alexa-Fluor 488 and goat anti-rabbit Alexa-Fluor 546, Life Technologies) and Alexa-Fluor 488 Phalloidin (1:100; Life Technologies) for F-actin. The nuclei were stained with

Hoechst 33342 (2 μg/ml; Life Technologies) for 5 mins at room temperature, followed by mounting sections with Vectashield (Vector Laboratories). Imaging was performed using a fluorescence microscope (Carl Zeiss; Axio Observer A1).

3.2.4 Real time-PCR

Total RNAs were extracted from cell-laden hydrogel (n=6) with Trizol, and reverse-transcribed into cDNA using the SuperScript Synthesis System (Invitrogen^{TM}). Real Time-PCR reactions were performed using the SYBR Green PCR Mastermix and the ABI StepOnePlus^{TM} Real-Time PCR system (Applied Biosystems, USA). cDNA samples (1 μl for a total volume of 20 μl per reaction) were analyzed for the genes of interest, and GAPDH was used as a reference gene. The level of expression of each target gene was then calculated as $-2^{\Delta\Delta Ct}$ as previously described [34]. Each sample was repeated at least three times for the gene of interest. The PCR primers are listed in table 1.

3.2.5 Statistical analysis

All Data are expressed as mean±standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA single factor) with * $p < 0.0$

3.3 Results

3.3.1 Viability of bovine chondrocytes

The cytotoxicity of the hydrogels was examined by a viability assay (Figure. 2.3B). Calcein-AM labeled (green) cells were used to monitor cell viability over 2 days of culture. As indicated by green fluorescent cytoplasmic labeling and negligible EthD-1 binding to nucleic acids (red) in maximum intensity projection, all scaffold compositions supported cell viability over two days of cultivation. Quantitative analysis of cell viability showed that only 14 % of encapsulated cells were dead, and the presence of RGD did not enhance the chondrocyte viability (Figure. 2.3). These results indicate that ECM-based hydrogels can support the cell survival regardless of integrin engagement.

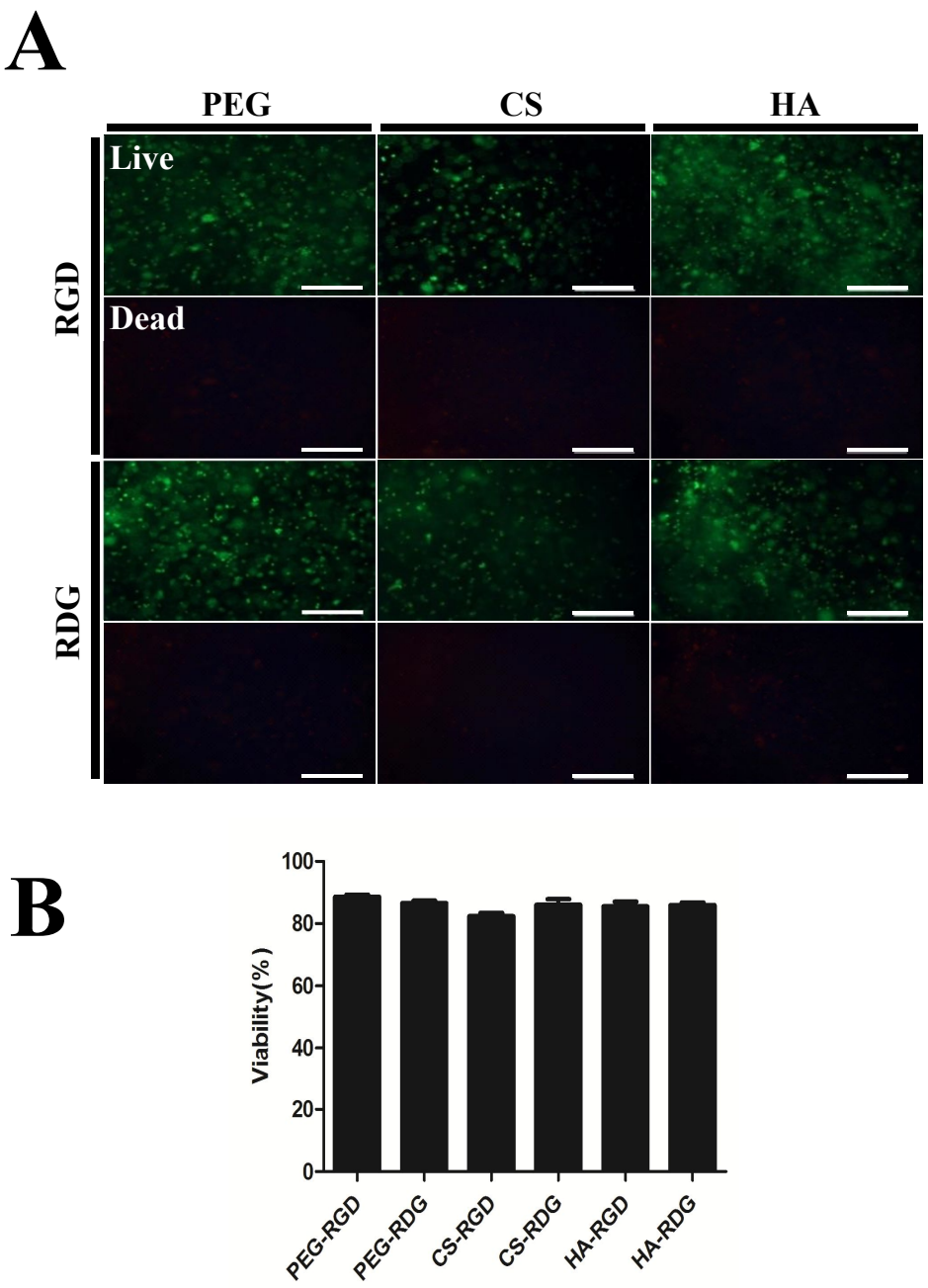

Figure 2.3 (A) Live/Dead viability Cytotoxicity kit was used for the viability test. Live cell was stained by Calcein AM and dead cell was stained by Ethd-1. Scale bar=200 μm. (B) Viability was measured by calculating the ratio of live cell and total cell number. Error bars represent the standard deviation on the mean for n=3.

3.3.2 Effect of ECM-based hydrogels on chondrocyte morphology, proliferation, and ECM synthesis

The relationship between cellular morphology and different types of ECM in 3D culture was next examined. Bovine chondrocytes were encapsulated in RGD/RDG-modified ECM-based hydrogels and maintained chondrocyte culture medium for 21 days. Constructs were fixed, and encapsulated cellular morphologies were evaluated by actin staining. Alexa Fluor® 594 Phalloidin staining revealed few micro-size protrusions of the cells into the surrounding matrix (White arrows in Figure 3.1). All ECM-based hydrogels presented uniformly distributed viable cells that displayed a rounded morphology. However, some cellular protrusions were only present in RGD-modified PEG-RGD, CS-RGD and HA-RGD group. From the result, we confirmed that contacts of cells with the surrounding ECM are mediated by cell adhesion receptors (RGD) in multicellular organisms. However, no strong correlations were observed between these cellular protrusions and matrix elasticity. DNA content or cell number after 3 weeks culture in CS-RGD hydrogel was 0.1415 % that this value is statistically higher than DNA content of PEG-RGD, which was only 0.0929 % (Figure. 3.2). Exogenous amount of CS and HA hydrogels produced significantly larger cell numbers compare to PEG-based hydrogels. Interestingly, cellular proliferation was not stimulated by the presence of RGD. Quantitative analysis of GAG indicated that cells in CS-RGD hydrogels accumulated the highest levels of GAG followed by CS-RDG. Bovine Chondrocytes in PEG-RGD accumulated the lowest GAG levels (only 20% of GAG levels found in CS-RGD; Figure. 3.2). Amount of collagen was significantly

higher in CS-RGD group compare to PEG-RGD of hydrogel, but difference between rests of the group made no odds (Figure. 3.2).

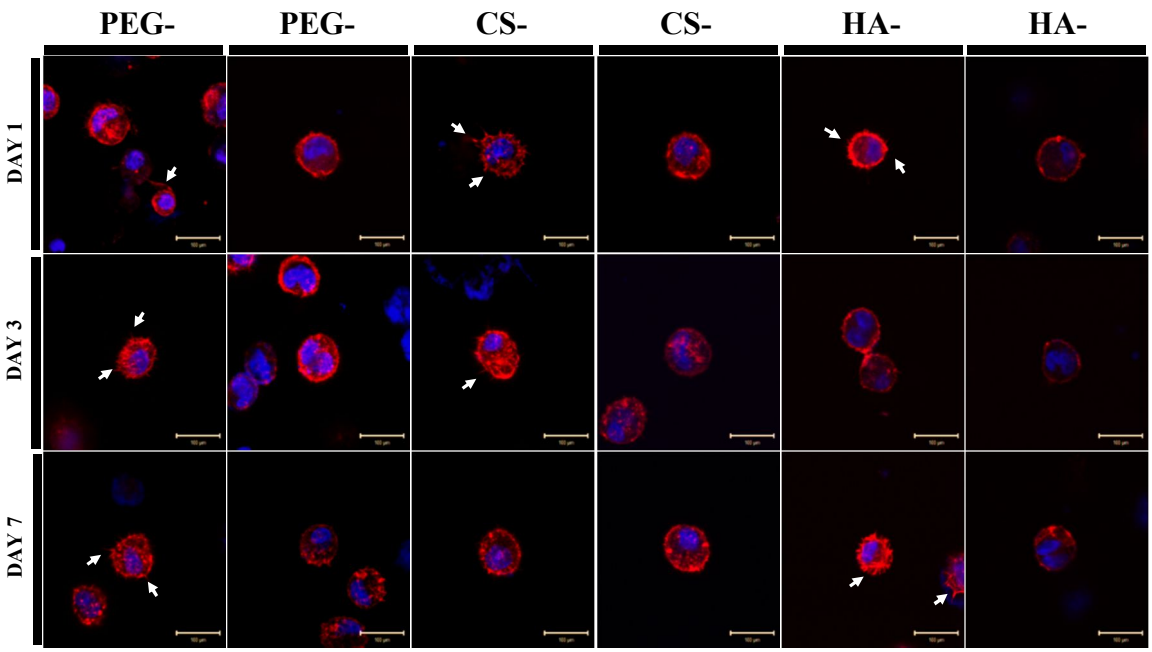

Figure 3.1 Morphological analysis and biochemical analysis of chondrocytes in RGD/RDG-modified ECM hydrogels. Each group of hydrogels was stained with Phalloidin and DAPI to observe actin cytoskeleton and nucleus. Scale bar = 100 μm.

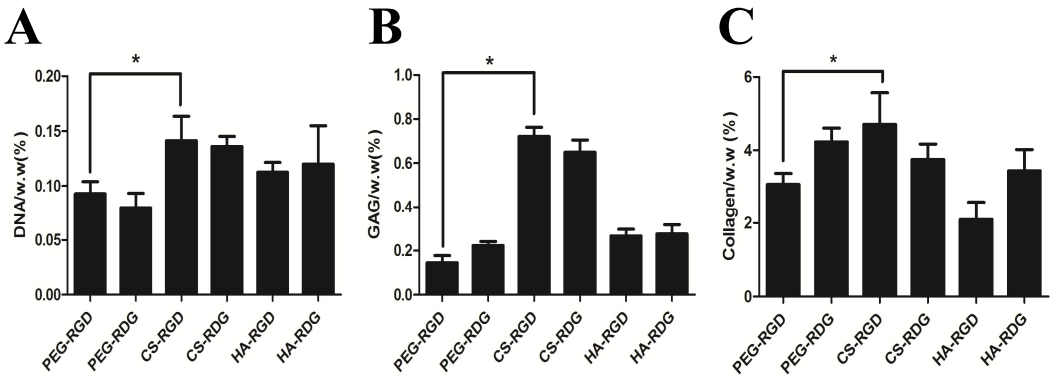

Figure 3.2 Quantification of cell proliferation (DNA) and matrix production (GAGs and Collagen) of cells were determined by biochemical assays. Error bars represent the standard deviation on the mean for n=3. *$p<0.05$

3.3.3 Histological and immunostaining analysis of engineered cartilage tissues

Histological evaluation of chondrocytes after 3 weeks of culture indicated that RGD group maintained higher matrix accumulation in the hydrogel. In addition, number of pericellular matrix visualized with H&E staining was higher in the RGD group compared to the RDG group (Fig. 3.3). Moreover, the diameter of pericellular matrix visualized by Safranin-O staining (red) was larger and vivid in the RGD groups of hydrogel, which demonstrates that accumulation of cell secreted proteoglycan was more apparent in RGD groups compared to RDG groups of hydrogel (Fig. 3.4). Even though CS-RGD and RDG hydrogels stained positive for Safranin-O due to CS content in the hydrogel, GAG contents around the cell appeared much intense in CS-RGD group (Fig. 3.4 middle). Immunostaining analysis for collagen type I and II demonstrated significant differences between RGD-modified hydrogels and RDG-modified hydrogels (Figure. 3.5 and 3.6). Cells encapsulated in RGD-modified hydrogels produced concentrated and well-defined cartilaginous ECM in the periphery of the cells as indicated by staining with type II collagen antibody (Fig. 3.6). Especially, CS-RGD group produced strong type II collagen network throughout the hydrogel, which closely mimic the natural tissue. Higher type I collagen production is indicative of dedifferentiation of the chondrocyte. However it was not detected throughout the RGD/RDG modified ECM-based hydrogels (Fig. 3.5).

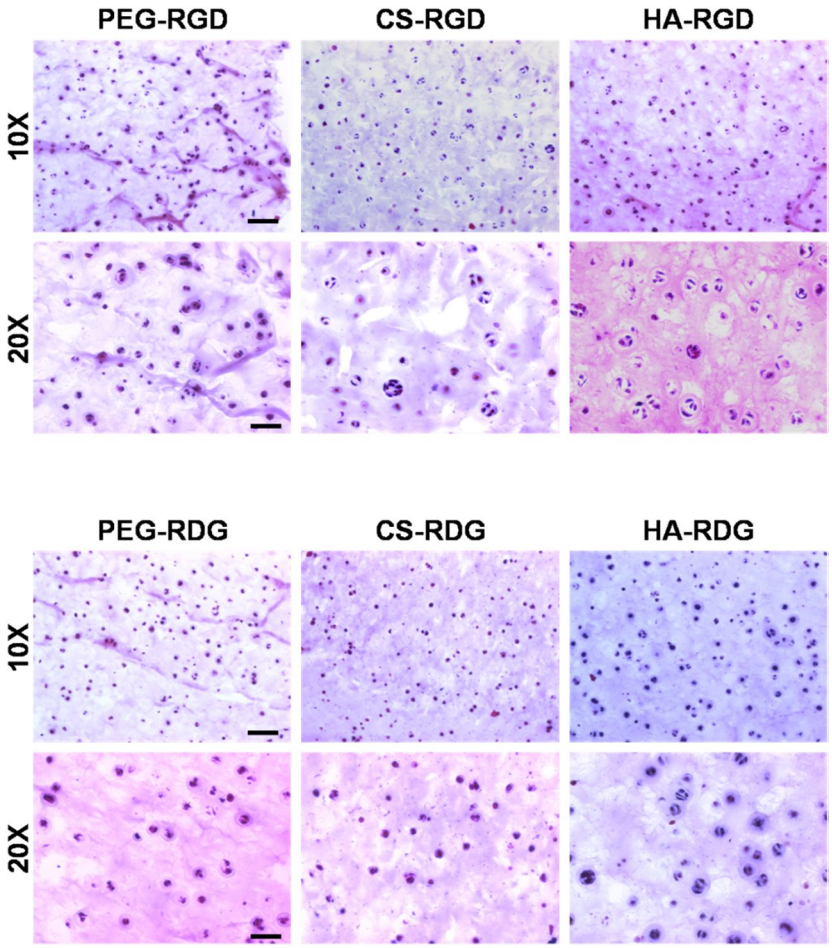

Figure 3.3 H&E staining of RGD/RDG-modified ECM-based hydrogels encapsulated chondrocytes at week 3 weeks. Hematoxylin & Eosin (H&E) staining demonstrates cellular morphology of RGD and RDG.

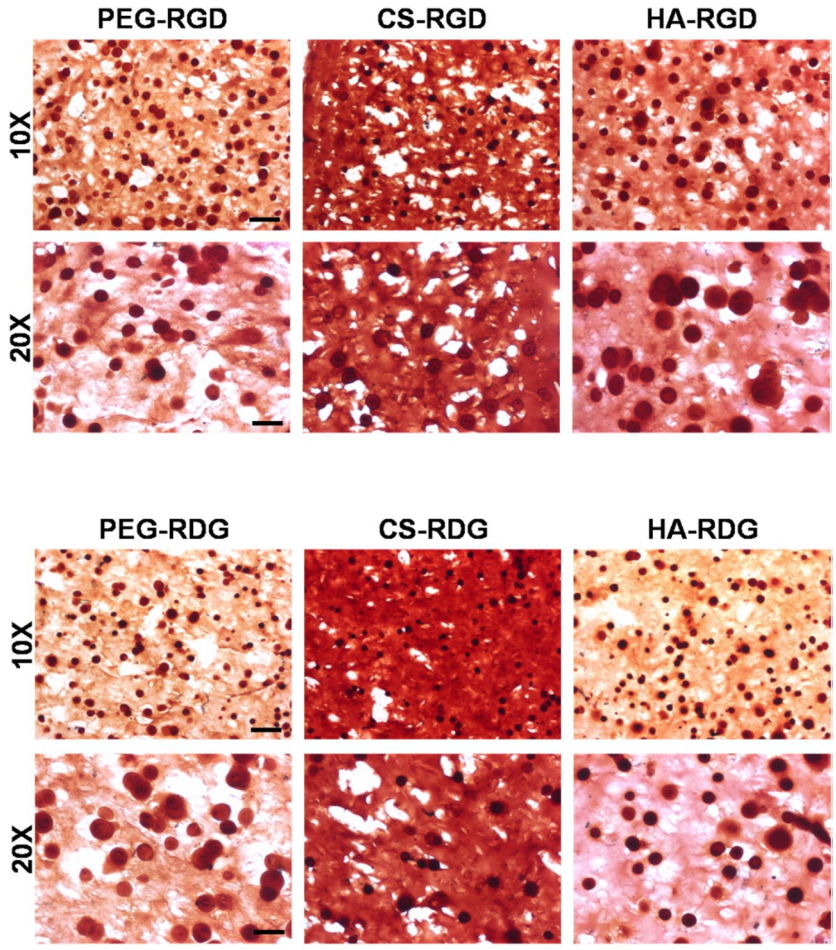

Figure 3.4 Safranin-O staining (Saf-O) demonstrates deposition of GAGs of RGD and RDG. Scale bar = 100 μm (10X) and 50 μm (20X), respectively.

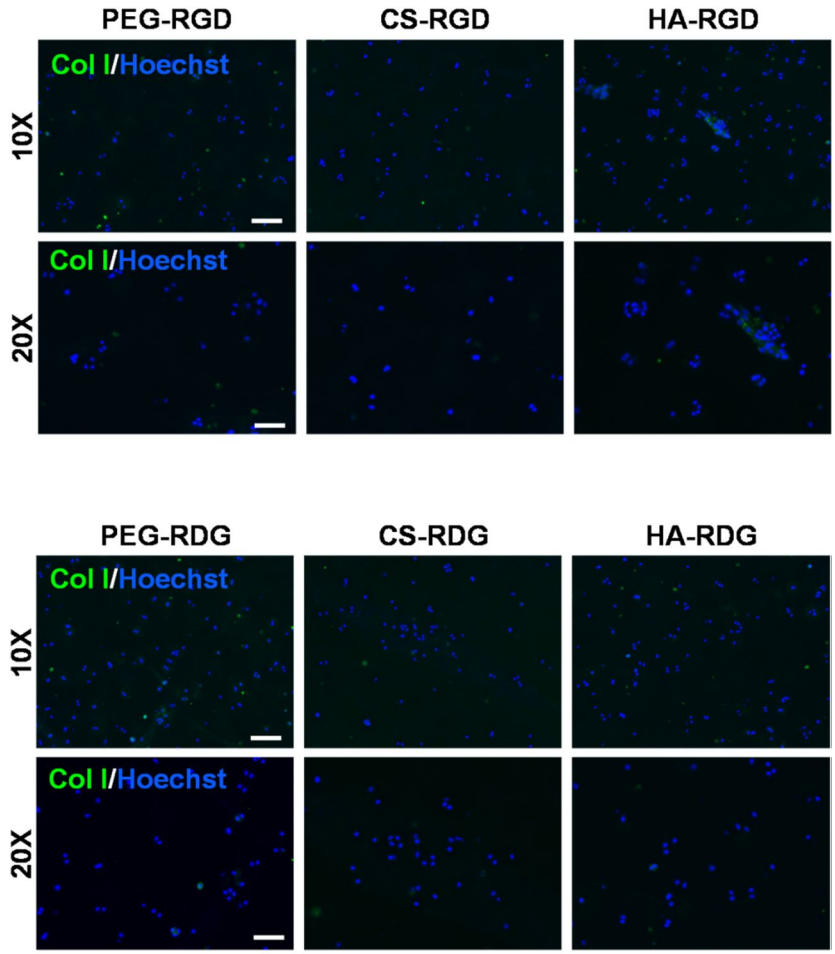

Figure 3.5 Immunofluorescence staining analysis. Collagen I staining (green) demonstrates dedifferentiation with Hoechest DNA staining (blue) of RGD and RDG.

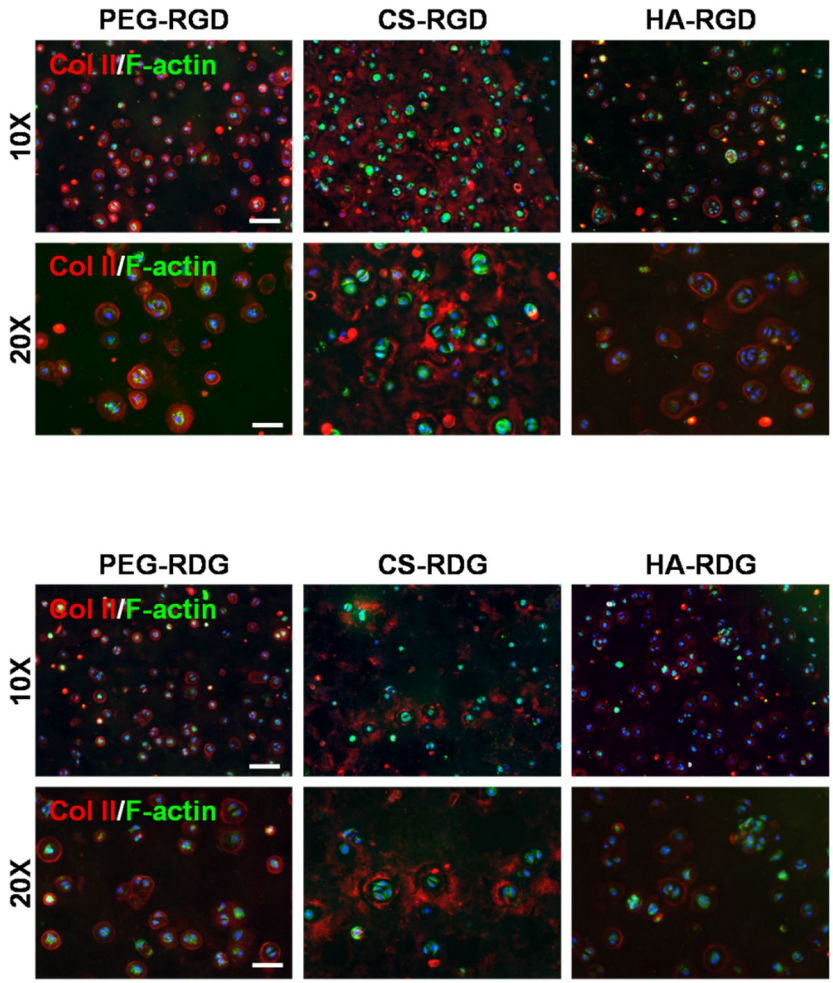

Figure 3.6 F-actin (green) and collagen type II (red) expression of RGD and RDG were assessed by dual immunofluorescence. Scale bar = 100 μm (10X) and 50 μm (20X), respectively.

3.3.4 Gene expression analysis of chondrocytes in RGD/RDG-modified ECM-based hydrogels

To determine the biological effects of ECM-based photopolymerizing hydrogel with RGD, we investigated cartilage specific gene expression such as Type II collagen, Aggrecan, Hyaluronan Synthase 2 (HAS 2), Lubricin (Lub), and Link protein in each hydrogel cultured for 3 weeks (Figure 3.7 A to E). All gene markers were normalized to PEG-RDG hydrogel for day 1 culture. The results showed that the expression of aggrecan, HAS2, and type II collagen were up-regulated in PEG-RDG compared to the PEG-RGD. On the contrary to this, incorporation of RGD into CS and HA, all three of cartilage relevant markers were up-regulated compare to its RDG groups. Thus, ECM production due to cell adhesion was activated only in the ECM included hydrogel. Real-time PCR analysis of aggrecan also matches with GAG biochemical assays in figure 3.2. For other two markers, lubricin and link protein, also CS-RGD is over-expressed compare to other groups. These results propose that RGD modification of biomaterial will enhance the chondrocyte's behavior in cartilage regeneration.

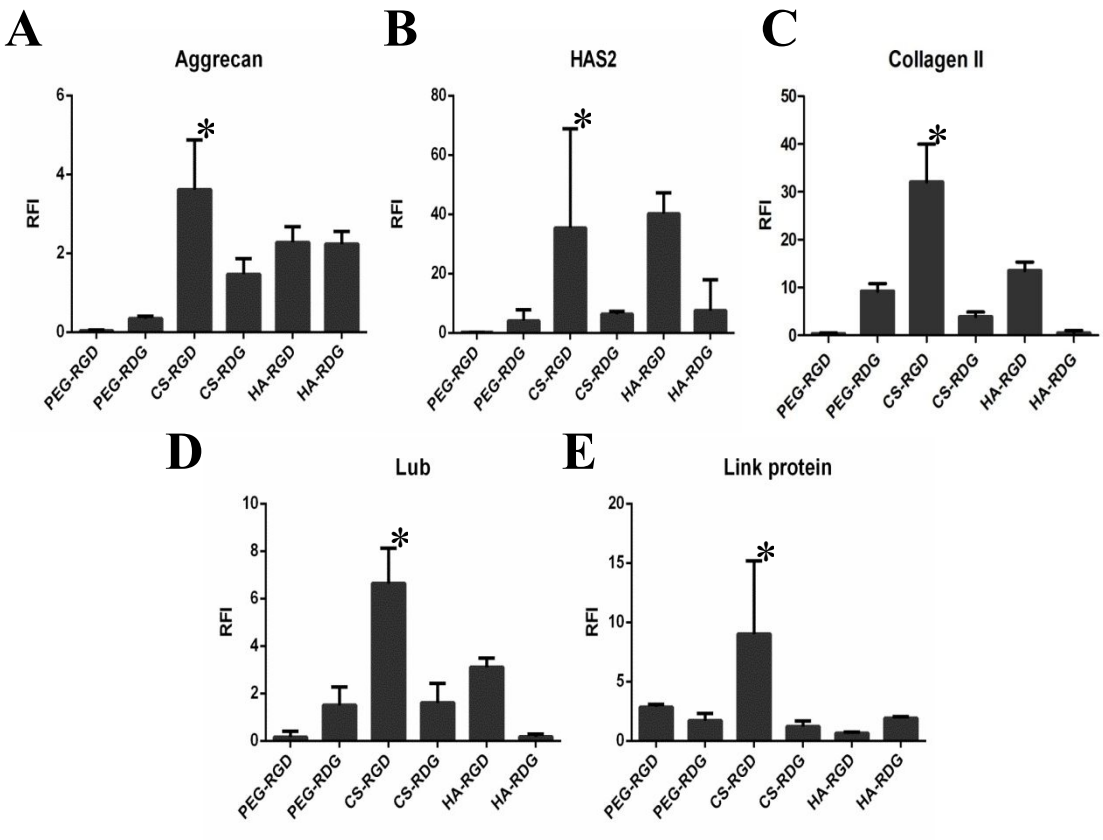

Figure 3.7. Analysis of gene expression level for Aggrecan (A), HAS2 (B), Collagen II (C), Lubricin (D) and link Protein (E) after cultured for 3 weeks. Gene expression was normalized by PEG-RDG hydrogel for 1 day culture and GAPDH was used for the housekeeping gene.

3.4 Discussion

RGD binds to α_v and β_1 integrin of chondrocytes and integrin activation leads to intracellular signaling pathway regulating cell-matrix interaction. These adhesion peptide and their receptors mediated inside-out signaling and outside-in signaling resulting in cell anchorage, differentiation, motility, migration and ECM remodeling in homeostasis [35-37]. However, in regards to chondrocytes in RGD microenvironment, previous studies showed conflicting results. Study by Levenston et al. showed that chondrogenesis of mesenchymal stem cells was inhibited by RGD containing hydrogel. They found that cell adhesive ligands inhibit stimulation of chondrogenic markers [38]. However, Anseth et al showed that RGD containing PEG-based hydrogels promotes cell survival and differentiation of mesenchymal stem cells [13, 39]. In addition, RGD conjugated chitosan hydrogel was used as a cell-supporting scaffold for articular cartilage regeneration [40]. The distinction may due to varying cellular phenotype and the composition of cellular microenvironments. From the experiment, RGD response in ECM microenvironment present a great contrast to RDG response because when integrin were activated through the RGD in ECM-based hydrogel, it may have modulated cellular behavior by surrounded microenvironment that consist of ECM molecule mimicking native environment. Of the ECM-based hydrogels examined, PEG-RGD and CS-RGD showed higher contents of DNA compare to RDG groups. Besides, CS-RGD has a greater amount of GAG contents over CS-RDG group. Therefore, distinctions in the cellular response to the scaffold were observed between most RGD and RDG binding hydrogels. Although RGD conjugation in CS and HA hydrogels showed activated ECM production.

Designing a proper scaffold, which could provide the necessary biological environmental to the encapsulated cells, is a pivotal issue in cartilage tissue engineering. In this study, we outlined a method for peptide (RGD/RGD) incorporation into ECD-hydrogels to permit engineered bio-specific cell adhesion in ECM-microenvironment. A finding of this study has yield in ECM-based hydrogels with notable cyto-compatibility and ability to provide a suitable microenvironment to produce cartilaginous tissues. In particular, we demonstrated here that the incorporation of host tissue-mimetic biomolecules along with covalently linked RGD peptide sequences significantly resulted in GAG-rich as well as collagen-rich tissues. Chondrocytes surrounded by their extracellular matrix maintain round morphology. Cell shape or actin organization plays an important role in cellular proliferation, cell differentiation and matrix synthesis [41, 42]. Previously, chondrocyte spreading due to high RGD concentration has shown to decrease cell size and reduce ECM synthesis overtime in polyethylene glycol dimethacrylate (PEGDM) hydrogel [43, 44]. Accordingly, in our study, slight decrease in GAG and collagen were observed compared to chondrocytes in PEG-RGD hydrogel compared to that of PEG-RDG hydrogels. Conversely, ECM-based hydrogels exhibited biodegradation properties upon exposure to their respective degradable enzymes [45, 46]. However, CS-based hydrogels showed faster degradation in vitro. Furthermore, immunostaining for type II collagen indicated that collagen accumulation in RGD-CS hydrogels was not limited to cellular periphery but detected throughout the hydrogel. Dynamic RGD-cell interactions in cell-degrading RGD-hydrogels probably may have contributed to increased GAG and collagen accumulation compared to other hydrogels.

Among many challenges facing cartilage tissue engineering is the needs for the production of functional proteins. In particular, among many functional proteins, lubricin, a mucinous glycoprotein encoded by the PRG4 gene, provides boundary lubrication in articular joints. Lubricating property plays an important role in the integration with surrounding tissue but optimal cell scaffold regulating this property has not been established. It has been demonstrated that lubricin secreting phenotype of chondrocyte is retained in the monolayer culture, whereas three-dimensional alginate hydrogel culture inhibited this phenotype [47]. Additionally recent study indicated that the extracellular matrix such as chondroitin sulfate, hyaluronic acid added alginate hydrogel induced lubricin expression [48]. Our findings were supported by this recent study as regards ECM molecule included hydrogel with cell adhesive characteristic enhanced lubricin secretion. CS microenvironment with RGD significantly enhanced lubricin and HAS gene expression. Strategies to engineer articular cartilage tissues should, therefore, focus on improving the expression of the lubricin and HAS genes, in analogy to functionality of articular cartilage. In addition, optimizing ECM-cell interactions may provide enhanced microenvironment for cartilage tissue engineering.

CONCLUSION

In this study, the influences of cellular microenvironments on cell-integrin interactions were investigated by examining viability, production of cartilage ECM macromolecules, and gene expressions of chondrocytes encapsulated within hydrogel. Furthermore, cellular morphology, as observed by the actin cytoskeletal structure of chondrocytes, was strongly dependent on the types of ECM components as well as the presence of integrin binding moieties in hydrogels. For articular cartilage tissue engineering, in addition to support of cellular phenotype, the expression of lubricin and hyaluronic acid synthase (HAS) remains to be a substantial challenge. In this regards, it was found that ECM-specific microenvironments and integrin interactions modulate the expression of lubricin and HAS genes of chondrocytes. Overall this study was able to outline an understanding of the interactions between chondrocytes and the ECM components along with integrin mediated cellular functions.

REFERENCE

[1] Loh QL, Choong C. Three-dimensional Scaffolds for Tissue Engineering: Role of Porosity and Pore Size. Tissue engineering Part B, Reviews. 2013.

[2] Shofty B, Ben-Sira L, Freedman S, Yalon M, Dvir R, Weintraub M, et al. Visual outcome following chemotherapy for progressive optic pathway gliomas. Pediatric blood & cancer. 2011;57:481-5.

[3] Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC, et al. Engineering vascularized skeletal muscle tissue. Nature biotechnology. 2005;23:879-84.

[4] Lee HJ, Yu C, Chansakul T, Varghese S, Hwang NS, Elisseeff JH. Enhanced chondrogenic differentiation of embryonic stem cells by coculture with hepatic cells. Stem cells and development. 2008;17:555-63.

[5] Tan F, Xu X, Deng T, Yin M, Zhang X, Wang J. Fabrication of positively charged poly(ethylene glycol)-diacrylate hydrogel as a bone tissue engineering scaffold. Biomedical materials. 2012;7:055009.

[6] Heinegard D, Saxne T. The role of the cartilage matrix in osteoarthritis. Nature reviews Rheumatology. 2011;7:50-6.

[7] Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials. 2005;26:5474-91.

[8] Tibbitt MW, Anseth KS. Hydrogels as extracellular matrix mimics for 3D cell culture. Biotechnology and bioengineering. 2009;103:655-63.

[9] Schmidt TA, Gastelum NS, Nguyen QT, Schumacher BL, Sah RL. Boundary lubrication of articular cartilage: Role of synovial fluid constituents. Arthritis & Rheumatism. 2007;56:882-91.

[10] Tibbitt MW, Anseth KS. Hydrogels as extracellular matrix mimics for 3D cell culture. Biotechnol Bioeng. 2009;103:655-63.

[11] Marcombe R, Cai SQ, Hong W, Zhao XH, Lapusta Y, Suo ZG. A theory of constrained swelling of a pH-sensitive hydrogel. Soft Matter. 2010;6:784-93.

[12] Annabi N, Tsang K, Mithieux SM, Nikkhah M, Ameri A, Khademhosseini A, et al. Highly Elastic Micropatterned Hydrogel for Engineering Functional Cardiac Tissue. Advanced functional materials. 2013;23.

[13] Nuttelman CR, Tripodi MC, Anseth KS. Synthetic hydrogel niches that promote hMSC viability. Matrix Biology. 2005;24:208-18.

[14] Becerra-Bayona S, Guiza-Arguello V, Qu X, Munoz-Pinto DJ, Hahn MS. Influence of select extracellular matrix proteins on mesenchymal stem cell osteogenic commitment in three-dimensional contexts. Acta biomaterialia. 2012;8:4397-404.

[15] Hwang NS, Varghese S, Zhang Z, Elisseeff J. Chondrogenic differentiation of human embryonic stem cell-derived cells in arginine-glycine-aspartate-modified hydrogels. Tissue Eng. 2006;12:2695-706.

[16] Hwang NS, Kim MS, Sampattavanich S, Baek JH, Zhang Z, Elisseeff J. Effects of three-dimensional culture and growth factors on the chondrogenic differentiation of murine embryonic stem cells. Stem cells. 2006;24:284-91.

[17] Lee JH, Lee HB, Andrade JD. Blood compatibility of polyethylene oxide surfaces. Progress in Polymer Science. 1995;20:1043-79.

[18] Knop K, Hoogenboom R, Fischer D, Schubert US. Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. Angewandte Chemie. 2010;49:6288-308.

[19] Kizilel S, Sawardecker E, Teymour F, Perez-Luna VH. Sequential formation of covalently bonded hydrogel multilayers through surface initiated photopolymerization. Biomaterials. 2006;27:1209-15.

[20] Smith DK. Supramolecular gels: building bridges. Nature chemistry. 2010;2:162-3.

[21] Hwang NS, Varghese S, Lee HJ, Theprungsirikul P, Canver A, Sharma B, et al. Response of zonal chondrocytes to extracellular matrix-hydrogels. FEBS Letters. 2007;581:4172-8.

[22] Hwang NS, Varghese S, Elisseeff J. Derivation of chondrogenically-committed cells from human embryonic cells for cartilage tissue regeneration. PloS one. 2008;3:e2498.

[23] Li Q, Williams CG, Sun DD, Wang J, Leong K, Elisseeff JH. Photocrosslinkable polysaccharides based on chondroitin sulfate. Journal of biomedical materials research Part A. 2004;68:28-33.

[24] Bryant SJ, Davis-Arehart KA, Luo N, Shoemaker RK, Arthur JA, Anseth KS. Synthesis and characterization of photopolymerized multifunctional hydrogels: Water-soluble poly(vinyl alcohol) and chondroitin sulfate macromers for chondrocyte encapsulation. Macromolecules. 2004;37:6726-33.

[25] Elisseeff J, McIntosh W, Anseth K, Riley S, Ragan P, Langer R. Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks. Journal of Biomedical Materials Research. 2000;51:164-71.

[26] Nguyen KT, West JL. Photopolymerizable hydrogels for tissue engineering applications. Biomaterials. 2002;23:4307-14.

[27] Drury JL, Mooney DJ. Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials. 2003;24:4337-51.

[28] Varghese S, Hwang NS, Canver AC, Theprungsirikul P, Lin DW, Elisseeff J. Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells. Matrix biology : journal of the International Society for Matrix Biology. 2008;27:12-21.

[29] Hwang NS, Varghese S, Li H, Elisseeff J. Regulation of osteogenic and chondrogenic differentiation of mesenchymal stem cells in PEG-ECM hydrogels. Cell and tissue research. 2011;344:499-509.

[30] Chung C, Burdick JA. Influence of three-dimensional hyaluronic acid microenvironments on mesenchymal stem cell chondrogenesis. Tissue engineering Part A. 2009;15:243-54.

[31] Kjellen L, Lindahl U. Proteoglycans: structures and interactions. Annual review of biochemistry. 1991;60:443-75.

[32] Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochimica et biophysica acta. 1986;883:173-7.

[33] Woessner JF, Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Archives of biochemistry and biophysics. 1961;93:440-7.

[34] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402-8.

[35] Meighan CM, Schwarzbauer JE. Temporal and spatial regulation of integrins during development. Current Opinion in Cell Biology. 2008;20:520-4.

[36] Giancotti FG, Ruoslahti E. Transduction - Integrin signaling. Science. 1999;285:1028-32.

[37] Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. Nature reviews Cancer. 2002;2:91-100.

[38] Connelly JT, Garcia AJ, Levenston ME. Interactions between integrin ligand density and cytoskeletal integrity regulate BMSC chondrogenesis. Journal of cellular physiology. 2008;217:145-54.

[39] Salinas CN, Anseth KS. The enhancement of chondrogenic differentiation of human mesenchymal stem cells by enzymatically regulated RGD functionalities. Biomaterials. 2008;29:2370-7.

[40] Park KM, Joung YK, Park KD, Lee SY, Lee MC. RGD-conjugated chitosan-Pluronic hydrogels as a cell supported scaffold for articular cartilage regeneration. Macromol Res. 2008;16:517-23.

[41] Glowacki J, Trepman E, Folkman J. Cell-Shape and Phenotypic-Expression in Chondrocytes. P Soc Exp Biol Med. 1983;172:93-8.

[42] Brown PD, Benya PD. Alterations in Chondrocyte Cytoskeletal Architecture during Phenotypic Modulation by Retinoic Acid and Dihydrocytochalasin-B Induced Reexpression. J Cell Biol. 1988;106:171-9.

[43] Vonwil D, Schuler M, Barbero A, Strobel S, Wendt D, Textor M, et al. An Rgd-Restricted Substrate Interface Is Sufficient for the Adhesion, Growth and Cartilage Forming Capacity of Human Chondrocytes. Eur Cells Mater. 2010;20:316-28.

[44] Smith Callahan LA, Childers EP, Bernard SL, Weiner SD, Becker ML. Maximizing phenotype constraint and extracellular matrix production in primary human chondrocytes using arginine-glycine-aspartate concentration gradient hydrogels. Acta biomaterialia. 2013;9:7420-8.

[45] Leach JB, Bivens KA, Patrick CW, Schmidt CE. Photocrosslinked hyaluronic acid hydrogels: Natural, biodegradable tissue engineering scaffolds. Biotechnol Bioeng. 2003;82:578-89.

[46] Kirker KR, Prestwich GD. Physical properties of glycosaminoglycan hydrogels. J Polym Sci Pol Phys. 2004;42:4344-56.

[47] Klein TJ, Schumacher BL, Blewis ME, Schmidt TA, Voegtline MS, Thonar EJ, et al. Tailoring secretion of proteoglycan 4 (PRG4) in tissue-engineered cartilage. Tissue Eng. 2006;12:1429-39.

[48] Coates EE, Riggin CN, Fisher JP. Matrix molecule influence on chondrocyte phenotype and proteoglycan 4 expression by alginate-embedded zonal chondrocytes and mesenchymal stem cells. Journal of Orthopaedic Research. 2012;30:1886-97.

Table 1.1 Primer list

Gene	Primer 5'-3'
GAPDH	F: TGT TGT GGA TCT GAC CTG CC
	R:TTC TCA GTG TGG CGG AGA TG
Type-II collagen	F:CTG TCC TCT GCG ACG ACA TAA
	R:CCA TCT GGG CAG CAA AGT TTC
Aggrecan	F:CGG TGA GAC GTC AGC CTA TC
	R:ATT CTG GGA TGG TGG TGC TG
Lubricin	F:CGC AGA AAC CAA CCA AAG CA
	R:TTG ATG CCG AAG CCT TGA CT
HAS2	F:GGA TTA TGT ACA GGT TTG TGA TTC AGA
	R:ACC TCC AAC CAT GGG ATC TTC
Link Protein	F:GCT CTG TGC AAT ATC CCA TC
	R:CCC ACT TTT GCA ATC TGA GC

요약(국문초록)

연골 조직 공학을 위한 아르지닌-글리신-아스파르트산 펩타이드와 세포외기질기반의 광중합성 하이드로겔 합성

관절연골의 손상은 인구의 고령화와 함께 지속적으로 증가하는 경향성을 보이고 있다. 그러나, 현재까지 연골의 전체적 재생 및 최소한의 기능 회복에 대한 치료법은 여전히 확립되어 있지 않다. 광중합 기반의 생체주입식 하이드로겔은 선행연구로부터 생체 적합성과 합성과정의 유동성, 구성 요소의 범위 및 물리적 특성의 이유로 연골조직공학에 적합한 재료로 인식되어왔다. 본 연구에서는 다양한 기능성의 생물작용을 내포한 광중합 기반의 하이드로겔을 폴리에틸렌 글리콜 디아크릴레이트 (이하, PEGDA), 세포외기질 (이하, ECM) 및 아르지닌-글리신-아스파르트산 펩타이드 (이하, RGD 펩타이드)와 함께 가교하여, 하이드로겔 내에서 고유한 생리 활성을 도입 및 유지 할 수 있도록 하였다. 따라서, 세포의 액틴 골격구조를 유심히 살펴 본 결과, ECM 및 RGD 펩타이드 존재 유무에 따라 세포의 형태가 주변 미세환경에 의존한다는 것이 밝혀졌다. 덧붙혀서, 여러 종류의 하이드로겔중, ECM 의 한 종류인 황산 콘드로이틴 (Chondroitin Sulfate, 이하 CS) 기반과 RGD 펩타이드가 혼합된 하이드로겔에서 소 연골세포의 가장 높은 Lubricin

의 유전자 증가를 보여주었다. 또한, 위에서 제시한 CS 기반의 하이드로겔은 다른 ECM 의 일종인 히알루론산 (Hyaluronic Acid, 이하 HA) 기반의 하이드로겔과 비교하여, 소 연골세포의 유전자 (DNA) 와, 글리코사미노글리캔 (Glycosaminoglycan, Gags) 및 콜라겐의 양이 증가 하였다. 이 연구 결과는 RGD 펩타이트와 ECM 의 미세 환경의 두 인자가 하이드로겔 기반 연골 조직 공학 응용을 위해 반드시 고려 되어야 함을 보여준다.

주요어: Hydrogel, Bovine Chondrocyte, SZP, RGD, Chondroitin Sulfate, Hyaluronic Acid, Cartilage Tissue Engineering

학번: 2012-23263