Abstract

Riboflavin induced photo-cross-linking of collagen hydrogel and its application in tissue engineering

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A meniscus tear is a common knee injury but its regeneration remains a clinical challenge. Recently, collagen-based scaffolds have been applied in meniscus tissue engineering. Despite its prevalence, application of natural collagen scaffold in clinical setting is limited due to its extremely low stiffness and rapid degradation. The purpose of the present study was to increase the mechanical properties and delay degradation rate of collagen-based scaffold by photo-crosslinking using riboflavin and UV exposure. Riboflavin is a biocompatible vitamin B2 that showed minimal cytotoxicity compared to conventionally utilized photo-initiator. Furthermore, collagen photo-cross-linking

with riboflavin improved mechanical properties and delayed enzymatic degradation rate of collagen scaffolds. Furthermore, in order to observe the meniscus tissue engineering, fibrochondrocytes were encapsulated. Photo-crosslinking of collagen resulted in reduced contractility and enhanced gene expression levels for the collagen II and aggrecan. Additionally, for activation of meniscus regeneration, hyaluronic acid (HA) was incorporated with collagen gel and showed increase of meniscus related gene level for the type I collagen, type II collagen, and aggrecan. Based upon these results, we demonstrate that photo-cross-linked collagen HA have potential for utilization in the scaffold-based meniscus tissue engineering.

Keywords: Collagen, Hydrogel, Riboflavin, Hyaluronic acid, Meniscus tissue engineering, Fibrochondrocyte

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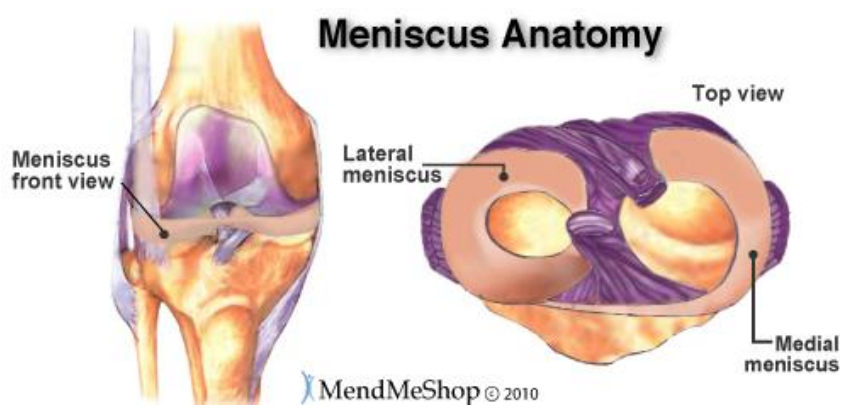
Chapter 1. THE SCIENTIFIC BACKGROUND AND RESEARCHS PROGRESS

1.1 Meniscus regeneration

Meniscus, a fibrocartilaginous tissue located between knee joints, greatly contributes to the knee health playing a critical role in shock absorption, load distribution and stabilization of the joint[1]. Meniscal tissues consist of water (72%), collagen fiber (22%) and proteoglycan (0.8%) and their biochemical composition varies depending on the region of the meniscus with different properties[2,3]. The outer meniscus is mainly consist of type I collagen which provides tensile force resistance, and the inner meniscus is consist of type II collagen and proteoglycan which promotes shock absorption function [4,5].



Meniscus tear is known to easily occur as aging or extreme exercising. When the meniscus tear occurs, the inner meniscus is unable to regenerate defects since it is an avascular region that lacks self-healing ability[6]. In the past, meniscus was generally considered as useless residue tissue generated during the joint formation. However, recent studies have revealed that the removal of meniscus may cause the development of degenerative arthritis in long-term and have emphasized its importance[7]. Numerous studies have been conducted to develop a treatment for meniscus tears. Some of major approaches are allograft transplantation, meniscectomy, and meniscus substitution[8,9]. Although such treatments have shown little improvements on regenerating the meniscus, the progress seem to be very limited with lack of qualified tissues, and both unstable and inefficient for long-term treatment. One of the promising alternatives is the scaffold-based tissue engineering. Scaffold-based tissue engineering provides cell-driven tissue regeneration through bioactive scaffold. For ideal meniscus tissue engineering, it is required to develop scaffold that fit properly to the meniscus. Scaffold which guides cells to regenerate functional tissue should be both biocompatible and able to induce synthesis of meniscal ECM component such as type I collagen, type II collagen and proteoglycan. Also, excessive degradation of scaffold should not be reached until complete tissue regeneration.

1.2 Collagen as a biomaterial

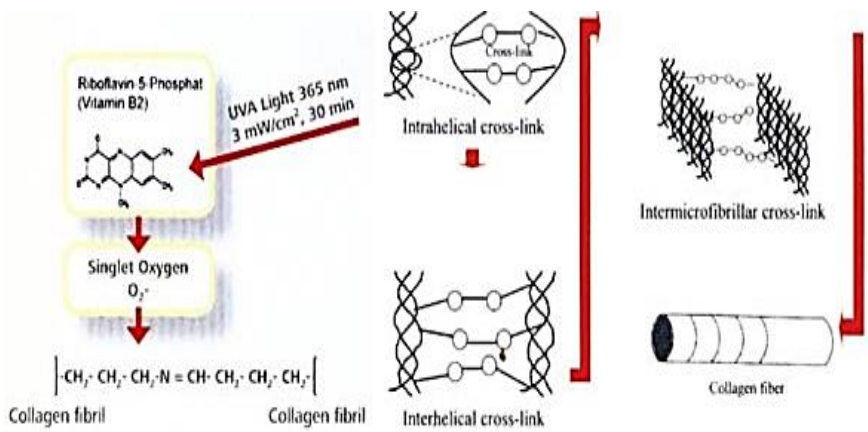
In tissue engineering, collagen is being widely utilized as the biomaterial in due to its abundance, biocompatibility and easy incorporation with new tissue matrix. Generally, collagen is denatured when the solvent is acidic and becomes hydrogel forming self-assembly triple helix structure at 37℃ with the neutral condition. Because collagen hydrogel is easily manufactured by controlling pH and temperature conditions, collagen based hydrogel have been commonly used in cell culture scaffold. In particular, type I collagen has been studied for biomaterial of meniscus scaffold since it accounts for 90% of collagen fiber existed in the meniscus[10,11].

However, due to weak mechanical property and rapid degradation, without the modification, it is difficult to handle collagen hydrogel clinically. Also, cell-collagen interaction leads to contraction of cell cultured scaffold caused by weak non-covalent bond. Such contraction property may work as the limitation of collagen gel by losing its original size and shape when used as defect filler. To overcome such limitation, various collagen cross-linking methods like chemical cross-link have been induced by glutaraldehyde, formaldehyde[12,13]. However, well-established methodologies are not yet developed for cell containing scaffold without cytotoxicity.

1.3 Collagen crosslinking with riboflavin

In this study, we have investigated riboflavin as a photo-sensitizer for photo-cross-linking of collagen hydrogel scaffold. Combined riboflavin and UV has been proven clinically for strengthening of collagen layer in the cornea repair[14,15]. The cross-

linking results from the covalent bond formation between amino acid of collagen fibril induced by singlet oxygen generated from light excited riboflavin[16]. Due to its ability to strengthen collagen with non-cytotoxicity and in situ gelling, researches have gradually utilized this method for development of cell encapsulations. Recent studies by Ahluwalia developed riboflavin induced collagen hydrogel and examined viability when fibroblasts and chondrocytes were encapsulated[17]. Furthermore, Min Lee have showed greatly improved neocartilage formation through riboflavin induced cross-linked type II collagen and methacrylated glycol chitosan with TGF-β[18]. However, as far as we know, this method has not been applied for meniscus cell-based tissue engineering yet.



1.4 Research aims

The aims of this study were to determine the optimal treatment condition of riboflavin for injectable cell-based collagen scaffold and biosynthetic activity of meniscus cells populated scaffold. In addition, cross-linked hyaluronic acid (HA) known to promote

meniscus regeneration was supplemented to the photo-cross-linked hydrogel as a form of small bead and was investigated for their potential value for matrix production within the scaffold[19]. We hypothesized that cell encapsulated photo-cross-linked collagen gel would have potential for utilization in meniscus regeneration and addition of cross-linked HA could accelerate synthesis of ECM component.

Chapter 2. PHOTO-CROSS-LINKING OF COLLAGEN HYDROGEL WITH RIBOFLAVIN

2.1 Introduction

Meniscus is located between knee joint playing a critical role in shock absorption. It is susceptible to be damaged for sports or sudden knee twist due to load. When meniscus tear occur, cell encapsulated hydrogel which fill defect for regenerating meniscus tissue can be one of the alternative. Although Type I collagen is the main component of the meniscus and collagen hydrogel is utilized as the biomaterial, it has limitation to use as the meniscus scaffold due to rapid degradation, weak mechanical property, and contractility. Thus, this study aimed to overcome limitation of collagen hydrogel introducing photo-cross-linking method and establish optimal manufacturing condition for meniscus scaffold. We used riboflavin as a photosensitizer for photo-cross-linked collagen hydrogel which have strong covalent bond between collagen fibril and verified meniscus regeneration when rabbit fibrochondrocyte encapsulated within hydrogel

2.2 Experimental

2.2.1 Materials

Bovine type I collagen 3mg/ml (Advanced biomatrix), Sodium hydroxide (NaOH, Sigma), 10x phosphate-buffered saline (PBS, Gibco), 1x phosphate-buffered saline (PBS, Gibco), Riboflavin 5’-phosphate sodium salt hydrate (Sigma), Fetal bovine

serum (FBS, Gibco), Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12, Gibco), Penicillin-streptomycin(Pen strep, Gibco), Non-essential amino acid (NEAA, Gibco), Vitamin C (Sigma), Deuterium oxide (Sigma), Type II Collagenase (Worthington), Live/dead Cell viability/cytotoxicity kit (Molecular Probes, L-3224), Optimal temperature cutting compound (OCT, Cell Path Ltd.), Mayer's hematoxylin (Dako), Safranin-O (Polysciencesm, Inc), Eosin Y (Sigma), M-MLV cDNA synthesis kit (Enzynomics)

2.2.2 Fibrochondrocytes isolation and culture

Fibrochondrocytes were isolated from New Zealand white rabbit as described previously[10]. In brief, meniscus were removed from rabbit knee joint and the inner meniscus was dissected by sterile razor blade. Minced meniscus pieces were treated 0.2% collagenase in DMEM/F12 supplemented with 10% FBS, 100U/ml pen strep for overnight. Isolated cells were then plated with DMEM/F12, 10% FBS, 100U/ml pen strep, 50 μg/ml vitamin C, 100 μM NEAA and incubated at 37℃ in 5% CO_2.

2.2.3 Photo-cross-linking of collagen gel and cell encapsulation

Collagen hydrogel with riboflavin was prepared by as follows. 1 part of 10X PBS were slowly added to 9 parts of collagen solution in 0.01N acetic acid. And then different amount of 1% w/v of riboflavin solution in 10x PBS was mixed to collagen and subsequently neutralized by 1M NaOH. The final concentration of riboflavin was 0.001, 0.006, 0.01, 0.02, and 0.1%. Hydrogels were incubated at 37℃ for 10 or 20min followed by exposed to UV light (3.5mW/cm2) for 1, 3, and 5min. Collagen

hydrogel without riboflavin as a control were produced by manufacturer's protocol and it was only incubated for 1 hour. For encapsulation of isolated fibrochondrocytes into hydrogel construct, cells were gently pipetted with neutralized collagen precursor solution at a concentration of 1×106 cells/construct. Next, cell mixed solution in an 8 mm diameter of cylindrical mold was photo-cross-linked using UV light (3.5mW/cm2) for 3 min following incubation for 20 min. In case of control group, it was only incubated for 1 hour. The constructs were cultured for 3 weeks at 37℃ with CO2 incubation in fibrochondrcyte culture medium.

2.2.4 CD measurement

CD spectra were measured for analyzing incubation time dependent triple helix structure of photo-cross-linked collagen gel. Circular dichroism detector (AppliedPhotophysics Chirascan Plus) equipped with 150-W xenon lamp was used to measure CD spectra and spectrum range was 210 to 250 nm.

2.2.5 Rheological property, swelling ratio, in vitro degradation

Viscoelastic property of each hydrogels were determined by carrying out rheology measurements using strain-controlled rotational rheometer (TA Instrument, ARES). Hydrogel disks were prepared in 8mm diameter. For frequency sweep measurements, the strain was kept at 0.2%, and the frequency was varied from 0.1 to 100 rad/s. The temperature was maintained at 30.0 ℃ during the measurement.

2.2.6 Swelling ratio

Each hydrogels were swollen in PBS for overnight and wet weight was measured after removing the surface water with weighing paper. And then dry weight of freeze-dried hydrogels was measured. Swelling ratio was calculated by following equation.

$$\text{Swelling ratio (Q)} = \frac{wet\ weight\ of\ the\ equilibrated\ hydrogel\ in\ PBS}{weight\ of\ the\ dried\ hydrogel}$$

2.2.7 Live/dead assay and contraction assay

Fibrochondrocyte encapsulated hydrogels were incubated for 5 hours and sliced with razor blade. Live/Dead Cell viability/cytotoxicity kit was used followed by the manufacture's protocol. Cellular images were collected by Zeiss LSM 720 confocal microscope. To determine the degree of contraction, cell-laden hydrogel size were measured at various times with an ImageJ software.

2.2.8 Degradation

To investigate enzymatic degradation degree of photo-cross-linked hydrogel, samples were treated with 10 U/ml of type I collagenase. Time course data were collected by measuring weight of remaining hydrogels (n=3) as changing fresh collagenase every day. Collagen hydrogel was used as a control.

2.2.9 Real time-PCR analysis

Gene expression of type I collagen, type II collagen, aggrecan were analyzed after 3 weeks culture within hydrogel constructs (n=3). Total RNA was extracted from each hydrogels with Trizol and reverse transcribed into cDNA using M-MLV cDNA

synthesis kit according to manufacturer's instructions. Using SYBR Green PCR Mastermix and ABI StepOnePlus™ Real time PCR system, cDNA was amplified by rabbit specific primer for type I collagen, type II collagen, aggrecan. GAPDH was used as a control and gene expression level was calculated as $-2^{\Delta\Delta Ct}$. The rabbit specific primers were listed below in table 1.

2.2.10 Histological analysis

After 3 weeks culture of fibrochondrocyte within hydrogels, constructs were fixed in 4% paraformaldehyde, snap-frozen in OCT embedding media cooled by liquid nitrogen and cryosectioned into 10 ㎛. For H&E stainig, samples were stained with hematoxylin for 3 min followed by Eosin Y staining for 1 min. For Safranin-O staining, sections were stained with 0.1% Safrnin-O solution after hematoxylin staining for 3 min.

2.2.11 Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical significance between groups was determined by Student's t-test using Microsoft Excel with * $p < 0.05$.

Gene	Primer 5'-3'
GAPDH	F: TCA CCA TCT TCC AGG AGC GA
	R: CAC AAT GCC GAA GTG GTC GT
Type I collagen	F:CTG ACT GGA AGA GCG GAG AGT AC
	R:CCA TGT CGC AGA AGA CCT TGA
Type II collagen	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

Table 1. Primer list

2.3 Results

2.3.1 Preparation of photo-cross-linked hydrogel

In order for the hydrogel matrix to maintain its shape, two step gelation method was used[17] (Fig. 1). First, neutralized collagen solution with riboflavin was incubated forming self-assembled triple helix which indicates intrahelical cross-link[20]. The incubation time was controlled to be 10 or 20 minutes to form a partial triple helix structure and as images shown, collagen solution remained fluidic property still after the incubation. Subsequently, sample was treated with UV (3.5 mW/cm^2) for covalent bond cross-linking between amino acids of collagen fibril. After the separation of hydrogels from the cylindrical mold, we observed hydrogels was maintained their shapes with solid properties.

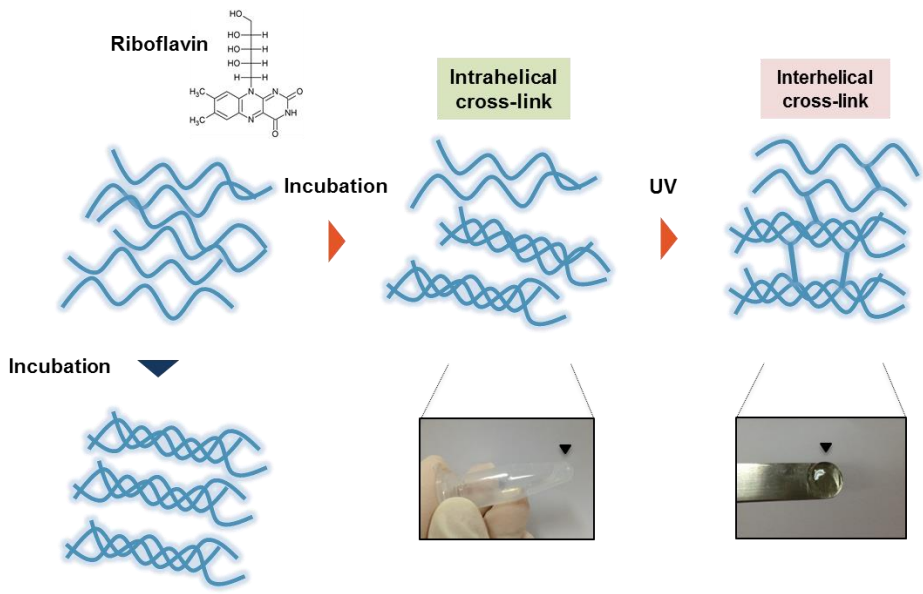


Figure 1. Schematic representation of photo-cross-linking of collagen with riboflavin. For the first step, collagen precursor solution were incubated to form intrahelical cross-link. Next, samples were UV treated to form interhelical cross-link between collagen fibril.

2.3.2 Dose dependent elastic modulus of hydrogel

To determine the appropriate riboflavin concentration to produce cross-linked hydrogels with improved mechanical property compared to non-cross-linked hydrogels (COL), RF concentration of 0.001, 0.005, 0.01, 0.02, and 0.1% were used for the frequency sweep measurements (Fig. 2A, B). In the figure 2A, the results indicated that photo-cross-linking with riboflavin could significantly increase elastic modulus of hydrogel than COL. Also, there was continuous increase of elastic modulus in the COL with greater dependency on frequency. In contrast, COL-RF exhibited consistent elastic modulus value at the range of frequency 0.1 to 100 Hz demonstrating more elastic behavior than COL. The results also suggest that the optimal concentration of RF concentration to increase the modulus of the constructs were 0.01% with 5.5 times stiffer than the COL (Fig. 2B). In this study, we optimized to the RF concentration of 0.01% with height of 3mm hydrogel.

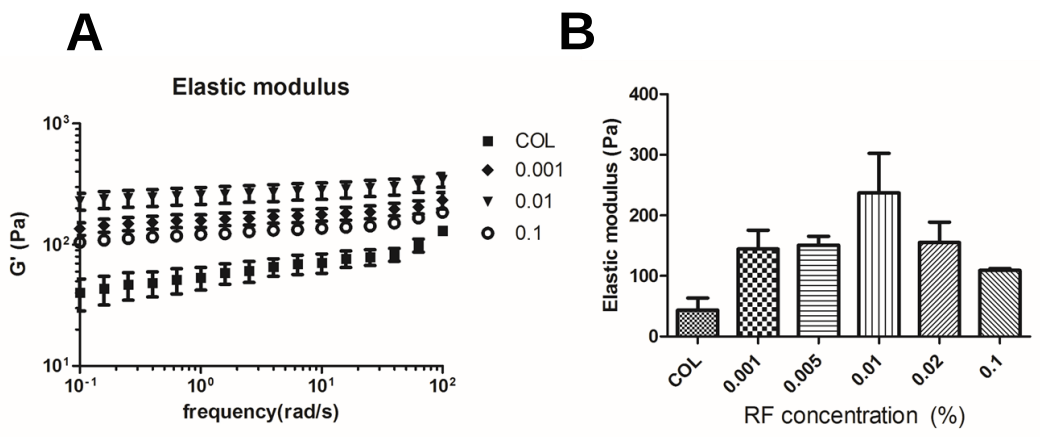


Figure 2. Elastic modulus of different final concentration of riboflavin mixed collagen hydrogel. Frequency sweep measurements were performed. Error bars represent the standard deviation on the mean for n=3.

2.3.3 Characterization of treatment time dependent photo-cross-linked collagen gel

We further examined the effect of two step gelation time. Treatment time was controlled as incubation for 10 or 20 minutes and UV exposure for 1, 3, and 5 minutes (Fig. 3A). As the incubation time shift 10 to 20 minutes, hydrogel became cloudy indicating more triple helix structure formation whereas UV exposure time did not influence the hydrogel appearance. To determine triple helix formation on the incubation for 10 min and 20 min, we carried out circular dichrosim (CD) measurements (Fig. 3B). Denatured collagen (collagen in acetic condition), COL, and COL-RF incubated for 10 or 20 minutes hydrogel groups were analyzed and UV exposure time was fixed to 3 minutes. COL, incubated for 90 minutes exhibited a negative peak at 190nm and a positive peak at 230nm which is the characteristics of triple helix structure of native collagen gel[21]. Similarly, CD spectra of incubation for 10 or 20 minutes indicated maximum peak at 230nm and minimum peak at 190nm. The results demonstrated that incubation for 10 or 20 minutes also have a triple helix structure.

The equilibrium swelling ratio of 6 hydrogel groups was measured (Fig. 4). COL had a swelling ratio of 104, which was higher than other COL-RF groups. Also, among COL-RF groups swelling properties did not show significant difference.

For discovering treatment time derived most efficient mechanical property, rheology test was performed (Fig. 5A, B). In the Fig 5A and 5B, incubation and exposure time was marked in the left and right side of the slash respectively. The elastic modulus was larger than the viscostic modulus and nearly constant

between a frequency of 1 and 100 Hz. As the incubation and exposure time increase, elastic modulus displayed a tendency to increase. In particular, elastic modulus of constructs was highly increased when incubated for 20 min and UV exposure for 3 and 5 min. However, there was no statistically significant difference with 6 groups.

Next, the biocompatibility of the scaffold depending on UV exposure time was examined by encapsulating rabbit fibrochondrocytes within COL-RF (Fig. 6A, B). COL-RF with isolated rabbit fibrochondrocytes was produced with UV exposure for 1, 3, and 5 minutes respectively, and viability assay was performed within 24 hours. Cell viability was approximately 93~94% for both 1 and 3 min and no significant difference were found between two conditions. However, when exposure for a longer period of time (5 min), cell viability dramatically decreased to 70%. Such result indicates that RF affects the cell viability when an irradiation exceeds 5 minutes. Thus, we progressed in further in vitro studies given 20 and 3 minutes of incubation and UV exposure time considering improved mechanical properties and higher viability.

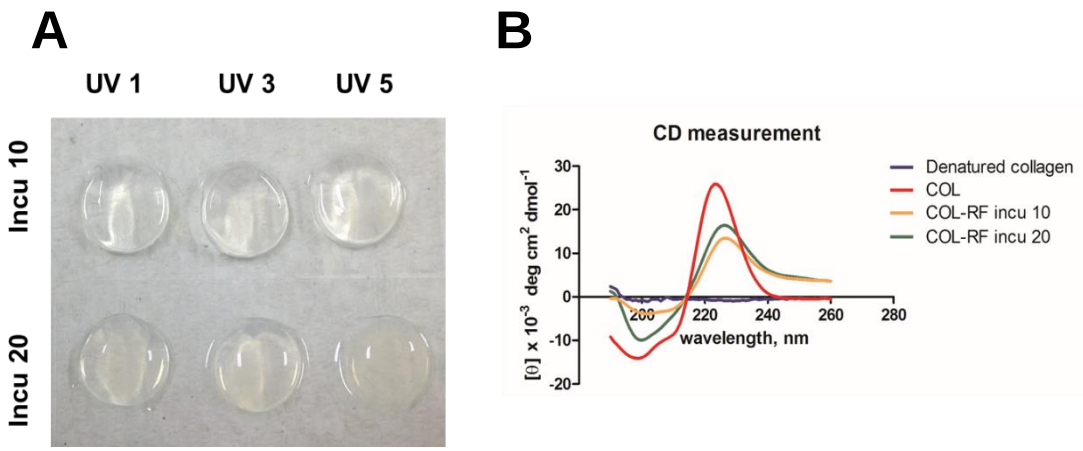


Figure 3. Incubation and UV exposure time dependent characterization of photo-cross-linked collagen hydrogel. (A) Gross images of acellular hydrogel. (B) CD measurement of denatured collagen (COL-acetic), collagen hydrogel without riboflavin (COL), and collagen hydrogel with riboflavin (COL-RF) incubated for 10 or 20 min.

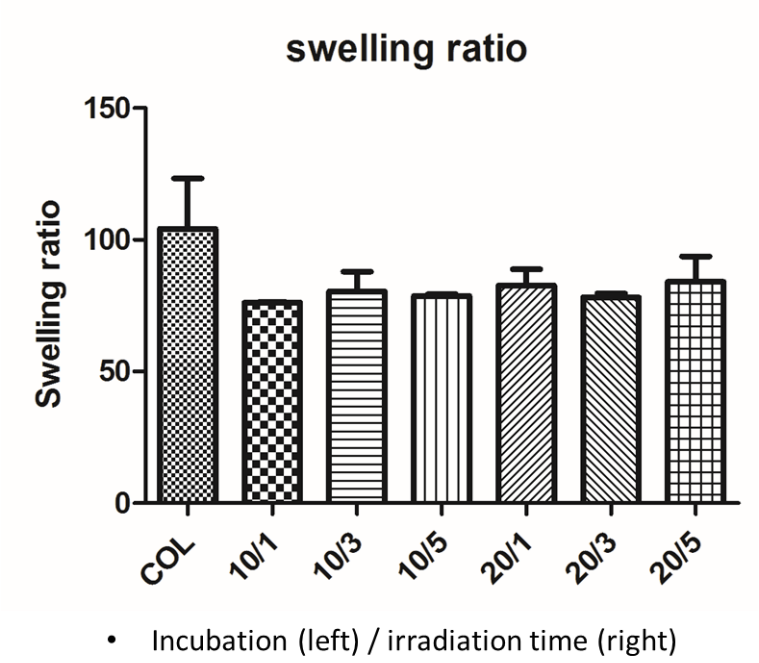


Figure 4. Swelling ratio of each hydrogel groups and collagen gel without riboflavin was used as a control. Error bars represent the standard deviation on the mean for n=3.

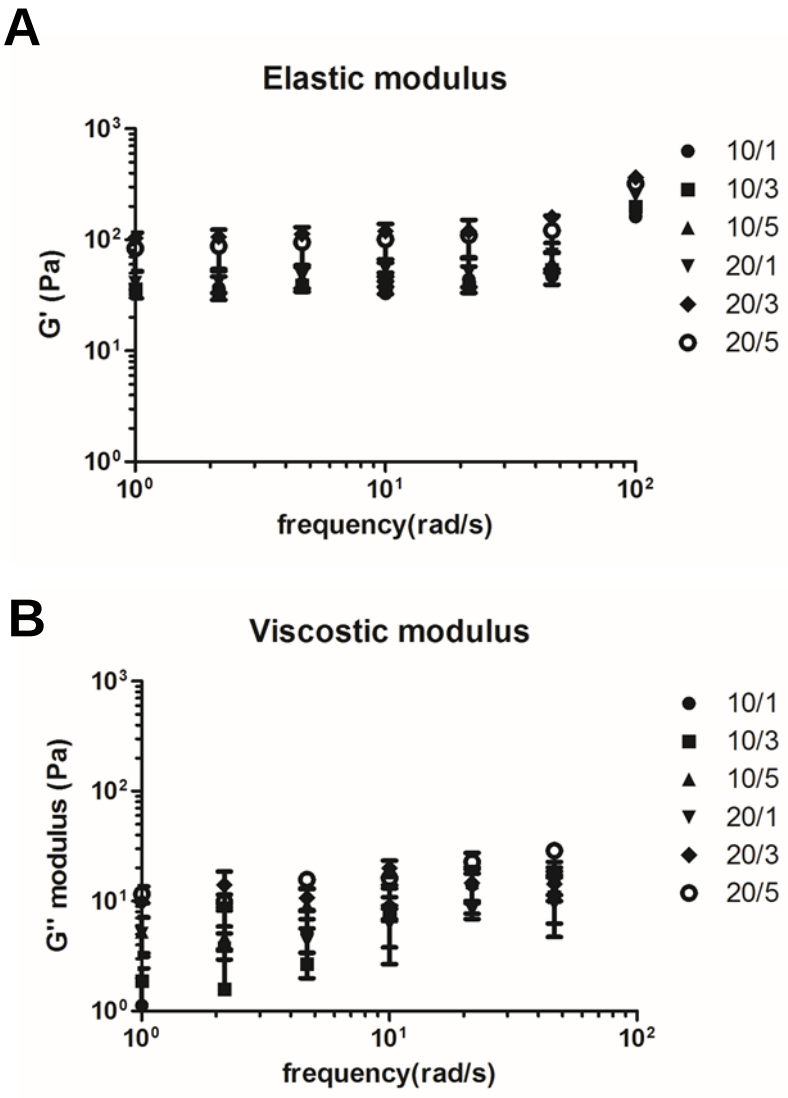


Figure 5. (A) Elastic modulus of the 6 hydrogel group with different treatment time. The left side of the slash means incubation time and the right side of the slash means UV exposure time. (B) Viscostic modulus of the 6 hydrogel group with different treatment time.

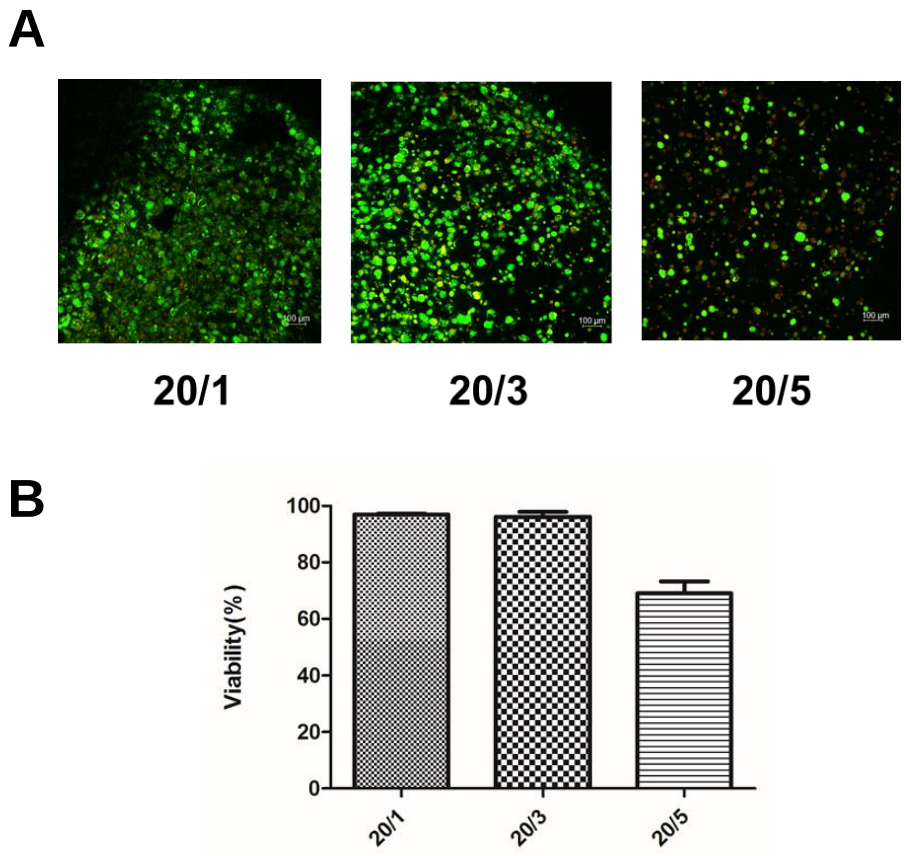


Figure 6. (A) Influence of UV exposure time on cell viability was assessed through Live/Dead viability Cytotoxicity kit. Live cells were stained green color by Calcein AM and dead cells were stained red color by Ethd-1. Scale bar=100 ㎛. (B) Viability was calculated by the ratio of live cell and total cell number. Error bars represent the standard deviation on the mean for n=3.

2.3.4 Contraction assay and degradation rate analysis

Spontaneous assembled collagen gel containing cells undergoes rapid contraction by cell–collagen interactions. In that regard, when collagen used as a tissue engineering scaffold, it could be another limitation along with degradation and weak mechanical property. To evaluate the contractility of the fibrochondrocyte contained collagen gel, we encapsulated 7×10^5 cells/construct of rabbit fibrochondrocytes (Fig. 7A, B). Initial diameter of constructs was 8mm and we observed the sudden contraction of COL to < 60 % of original size within 4 hours. In contrast, we found out that COL-RF retain 90 % of its original size even after 66 hours.

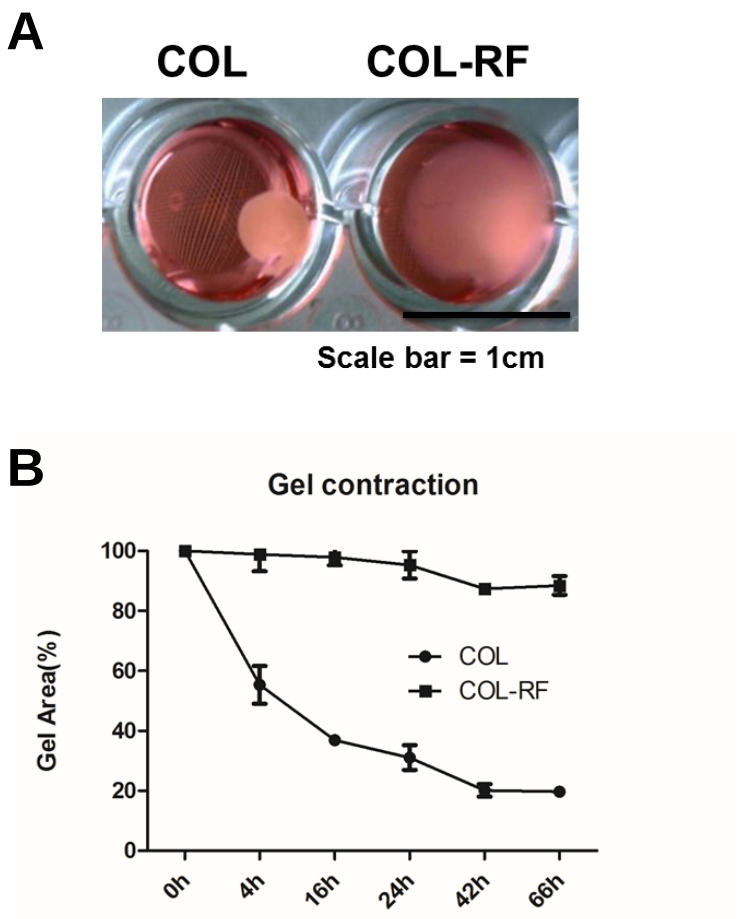


Figure 7. (A) Appearance of contracted hydrogels containing fibrochondrocyte after 4 hours encapsulation. (B) Comparison of contraction rate between collagen hydrogel with photo-cross-linking and without photo-cross-linking. The change of gel area was analyzed by measurement of the maximum gel diameter. Error bars represent the standard deviation on the mean for n=3.

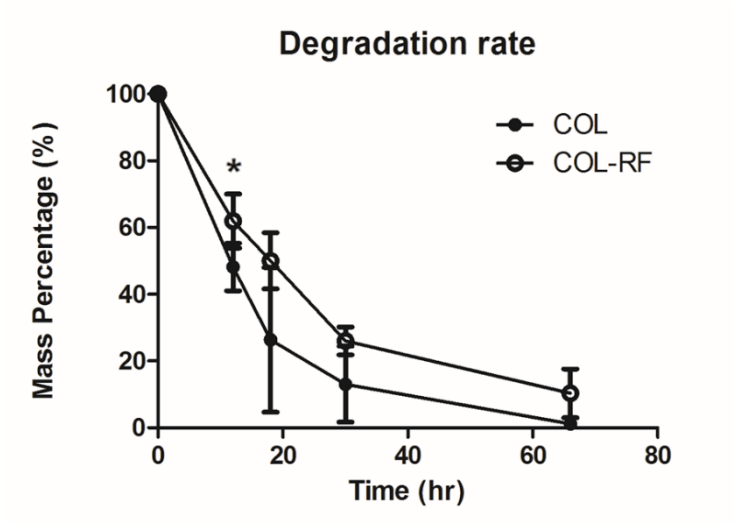


Figure 8. Effect of riboflavin induced photo-cross-linked collagen gel in enzymatic degradation. After treatment with type I collagenase, the mass of remaining construct was measured. Error bars represent the standard deviation on the mean for n=3.

2.3.5 Gene expression analysis and histological staining of fibrochondrocytes in COL and COL-RF

In order to examine the activity of fibrochondrocytes function that is production of ECM molecule within hydrogel, we performed gene expression analysis through real-time PCR. Isolated rabbit fibrochondrocytes (P2) were cultured for 3 weeks within COL and COL-RF. Investigated meniscus related gene was type I collage, type II collagen and aggrecan (Fig. 9). They are known as the component of the meniscus and we examined whether cells encapsulated in each hydrogel could induce production of this ECM material[22]. As the result, COL-RF showed decreased expression of type I collagen compared to control group with no significant differences. In contrast, COL-RF showed higher expression level of type II collagen and aggrecan compared to COL.

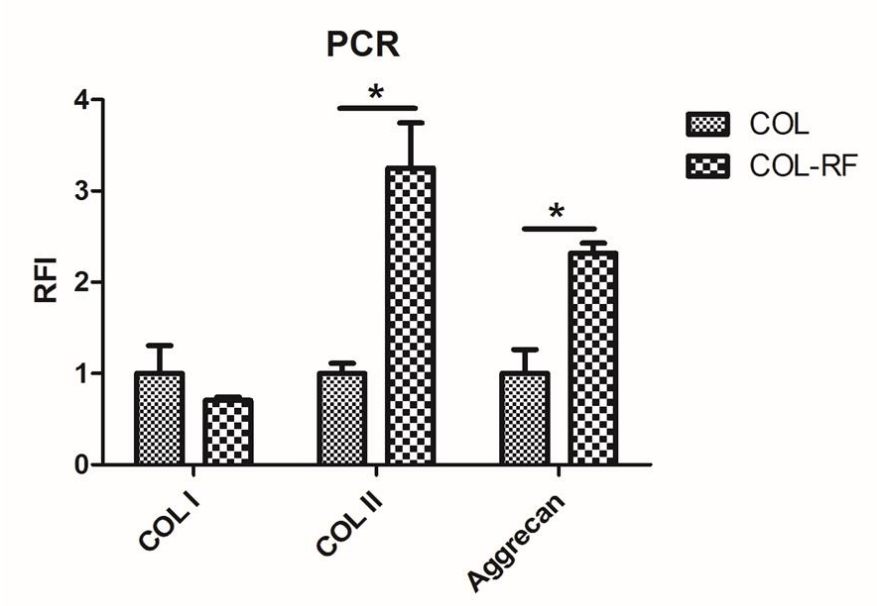


Figure 9. Relative gene expression level for the meniscus related factors, collagen I, collagen II, aggrecan after 3 weeks of rabbit fibrochondrocyte culture within each construct. Gene expression was normalized by collagen hydrogel of 1 day culture. Error bars represent the standard deviation on the mean for n=3.

2.3.6 Histological staining of fibrochondrocytes in COL and COL-RF

For evaluation for production of ECM matrix, histologic sections of each hydrogels populated with 3 weeks cultured fibrochondrocytes were stained by H&E and Saf-O (Fig. 10). H&E staining exhibited nuclei were evenly distributed throughout the both COL and COL-RF. Whereas two groups were presented distinction in the Saf-O staining. In the COL-RF, we identified the size of Saf-O (red) stained pericellular matrix was larger than COL, which represents greater deposition of GAG around the cells.

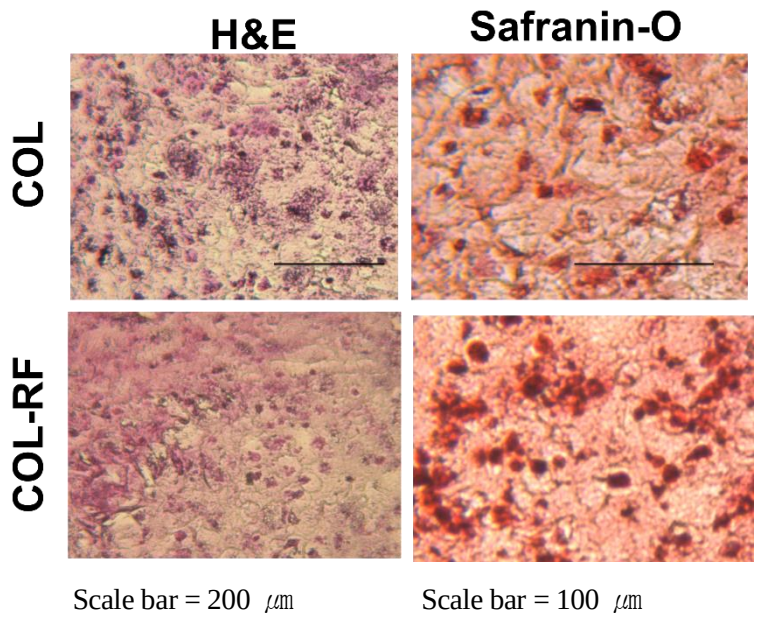


Figure 9. H&E staining and Safranin-O staining (Saf-O) of rabbit fibrochondrocyte populated hydrogel with 3 weeks culture.

2.4 Discussion

Although Type I collagen is the main component of the meniscus and collagen hydrogel is widely utilized as the biomaterial, it has limitation to use as the meniscus scaffold due to rapid degradation, weak mechanical property, and contractility. To overcome this limitation of collagen hydrogel, we introduced RF utilized photo-cross-linking method on collagen hydrogel. RF serves as a photo-sensitizer inducing production of oxygen radicals which mediate strong covalent bond between amino acid of collagen fibril[23, 24]. In this study, we hypothesized that RF induced photo-cross-linked collagen hydrogels are available for a meniscus cell populated scaffold due to non-cytotoxicity and short gelation time compared to other collagen cross-linking method. We aimed to establish optimal manufacturing condition for meniscus scaffold and verify meniscus regeneration when rabbit fibrochondrocyte encapsulated within hydrogel.

Rheological studies on COL and COL-RF (Fig. 2A, Supplementary Figure 1) revealed that the COL-RF had strong cross-link network which could cover environmental impact compared to COL with weak non covalent bond as previously reported by Rich. H[25]. The increase of cross-link density could be confirmed in swelling ratio, too. We identified COL-RF had a lower swelling ratio than COL. It represents a smaller space size and contained less amount of water in COL-RF. It may have been caused by dense COL-RF networks which have a lot of inter helical cross-link compared with COL which just have intra helical cross-link. Interestingly, elastic modulus of hydrogel rather showed decrease when RF concentration are exceeded 0.01%. Recent study indicated that increasing the riboflavin concentration above specific concentration did not lead to improve modulus of photo-cross-linked

MeGC/HA[26]. However to define why riboflavin have optimal concentration at 0.01% which is different concentration (0.1%) adapted for cornea cross-link, further studies should be investigated for the cross-link mechanism.

A finding of this study has yielded that cell viability was markedly decreased when fibrochondrocytes within COL-RF were exposure to UV over 5 min. Although RF exists in human bodies originally, due to free radicals generated during UV treatment, it may affects the cell viability in the longer irradiation time. Considering viability and elastic modulus, we designed photo-cross-linked collagen gel for meniscus scaffold with 0.01% of riboflavin concentration, 20 min of incubation and 3 min of UV exposure time.

Spontaneous assembled collagen gel containing cells undergoes rapid contraction by cell–collagen interactions. In that regard, when collagen used as a tissue engineering scaffold, it could be another limitation along with degradation and weak mechanical property. Ibusuki et al. has shown that the photo-cross-linking can reduce the contraction of COL containing fibroblast or chondrocyte keeping long-term cell viability[27]. Similarly, in our study, it appeared that photo-cross-linking technique on collagen populated with fibrochondrocytes could prevent the contraction. Furthermore, collagen hydrogel is known to be susceptible to enzymatic degradation. In this study, COL-RF retained mass much longer than COL in the enzymatic environments. Study by R.Zeeman and colleagues have shown that enzymatic degradation can be correlated with interfibrillar and intrafibrillar cross-links and their density[28]. Thus, the observed result demonstrated COL-RF have stronger bindings between fibrils than COL. Accordingly, COL-RF is expected to have potential to be

used in applications of a meniscus tissue engineering scaffold with well-maintained hydrogel shape and slower degradation rate.

We first expected that COL-RF can promote bio activation of fibrochondrocytes resulted from consistent cell-scaffold interaction due to less degradation. However, it was revealed that COL-RF could stimulate expression of type II collagen and aggrecan, but did not have any effect with expression of type I collagen. It is known that in the load-bearing tissue like meniscus, cell function was regulated by mechanical signal[29]. The increase of stiffness in the COL-RF may have caused promotion of gene expression level of type II collagen and aggrecan. Although Type II collagen network is the part of the inner meniscus along with proteoglycan, type I collagen is the main component which accounts for 90 % of collagen in the meniscus[30]. Since meniscus structure is highly organized together with type I collagen, type II collagen, and proteoglycan, COL-RF was not sufficient to be a scaffold promoting meniscus regeneration[31, 32].

Chapter 3. PHOTO-CROSS-LINKED COLLAGEN GEL WITH HYALURONIC ACID

3.1 Introduction

Hyaluronic acid (HA) is the naturally present in the body known to regulating bioactive reaction. In particular, recent studies revealed that HA could lead to meniscus healing, in the previous study, COL-RF showed the increase of GAG synthesis but did not have any effect on the type I collagen secretion of fibrochonodrocytes. Thus, we hypothesized that the addition of HA to COL-RF would help meniscus regeneration as supplementing bioactive effect to fibrochondrocytes. Moreover, to overcome rapid release of HA from hydrogel, we synthesized cross-linked HA. HMDA was used as the cross-linker of HA for synthesizing bulky molecule.

3.2 Experimental

3.2.1 Material

Sodium hyaluronate 64kDa (HA, Lifecore Co.), Hexamethylenediamine (HMDA, Sigma), 1-Ethyl-3-[3-(dimethyl amino)propyl]carbodiimide (EDC, Thermo scientific), 1-hydroxybenzotriazole monohydrate(HOBt, GL Biochem Ltd.), Carbazole, D-glucuronic acid, H2SO4, Hyaluronidase from bovine testes (Sigma), Mayer's hematoxylin (Dako), Safranin-O (Polysciencesm, Inc), Eosin Y (Sigma), M-MLV cDNA synthesis kit (Enzynomics), Bovine type I collagen 3mg/ml (Advanced

biomatrix), Sodium hydroxide (NaOH, Sigma), 10x phosphate-buffered saline (PBS, Gibco), 1x phosphate-buffered saline (PBS, Gibco), Riboflavin 5'-phosphate sodium salt hydrate (Sigma), Fetal bovine serum (FBS, Gibco), Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12, Gibco), Penicillin-streptomycin(Pen strep, Gibco), Non-essential amino acid (NEAA, Gibco), Vitamin C (Sigma), Deuterium oxide (Sigma)

3.2.2 Synthesis of cross-linked HA

Cross-linked HA was prepared as described[30]. Briefly, 4 w/v % of HA in distilled water was mixed with HMDA. The molar ratio of HMDA to carboxylic group of HA was 1:1. EDC and HOBt, were added to the mixed solution of HA and HMDA followed by incubating at 37℃ for 2hr. After completing cross-linking reaction, hydrogels were dialyzed against 1x PBS for 3 days eliminating unreacted material and freeze-dried for 2 days. Dried hydrogels were stored at -20℃ broken down to powder. In order to confirm cross-linking of HA, hydrogels were degraded by hyaluronidase (100U/mL) and then ^{1}H NMR analysis were performed.

3.2.3 Photo-cross-linking of collagen gel and cell encapsulation

Dried cross-linked HA was crush into less than 1mm by pestle and mortar. For HA laden collagen hydrogel, broken down HA particle were added to neutralized collagen-riboflavin precursor solution as previously described and the final concentration of HA was 1% w/v. The construct (Diameter 8mm, height 3mm) was incubated for 20 min at 37℃ and exposured to UV for 3 min sequentially.

3.2.4 Carbazole assay

To determine retention of cross-linked HA within hydrogel, release amount of cross-linked HA was measured by carbazole assay. Non-cross-linked HA was used as the control. Final concentration of 1% w/v of non-cross-linked or cross-linked HA within collagen hydrogel was incubated in the distilled water. Water was collected after 6 days and carbazole assay was performed as described previously using known concentration of D-glucuronic acid as standards[31].

3.2.5 Fourier-Transformed Infra-Red Spectroscopy (FT-IR)

FT-IR spectra of each hydrogels was obtained to determine the retention of HA in the collagen hydrogel. Using attenuated total reflection infra-red spectrometer (ATR-FTIR, Bruker Tensor27) at range of 4000 to 650 cm^{-1}, spectra were recorded. For comparison, freeze-dried cross-linked HA and collagen hydrogel were also analyzed.

3.2.6 Real time-PCR analysis

Gene expression analysis of each hydrogels was conducted by real time-PCR analysis for type I collagen, type II collagen and aggrecan. After 3 weeks of culture, total RNA was extracted from each constructs by Trizol and according to instructions, reverse transcribed into cDNA using M-MLV cDNA synthesis kit. cDNA was amplified by rabbit specific primer for type I collagen, type II collagen, aggrecan with SYBR Green PCR Mastermix and ABI StepOnePlusTM Real time PCR system. GAPDH

was used as the reference gene and gene expression level was calculated as $-2^{\Delta\Delta Ct}$. The sequence of rabbit specific primers were equal to the previous study (table 1).

3.2.7 Subcutaneous implantation of hydrogel

Fibrochondrocytes populated COL-RF and COL-RF-HA (1 million cells/construct) were cultured for 1 week in vitro and then inserted to 6 week nude mice. Mice were anaesthetized and disinfected with iodine. 3 cm of longitudinal incisions were made by surgical blade. Then, COL-RF and COL-RF-HA were implanted under subcutaneous pockets created by blunt dissection with scissors. The wounds were closed using 6/0 AILEE Co. Ltd.

3.2.8 Histological staining

After 4 weeks of in vivo culture, subcutaneously implanted hydrogels were harvested and fixed in 4% paraformaldehyde. After fixation, constructs were embedded in paraffin followed by dehydrated in ethanol. 4 μm thick sections were sliced and stained with haematoxylin and eosin or 0.1% Safranin-O.

3.2.9 Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical significance between groups was determined by Student's t-test using Microsoft Excel with * $p < 0.05$.

3.3 Results

3.3.1 Synthesis of cross-linked HA

HA was introduced to the collagen hydrogel for complement advantageous bioactive factors for meniscus regeneration. Also, in order to continuously retain HA in the COL-RF, cross-linked HA with HMDA was used (Fig. 11A). With supports from EDC and HOBt, HMDA was reacted as a cross-linker of carboxylic acid within the HA repeating unit. And at the end of the reaction, products were dialyzed, freeze-dried and split into powder form. Powder formed cross-linked HA was mixed with collagen precursor solution and incubated for 20 minutes and UV irradiated for 3 minutes. HA incorporated within COL-RF (COL-RF-HA) was formed in a shape of small bead, that was visible with naked eye. To confirm synthesis of cross-linked HA, dried cross-linked HA was digested by 100U/ml of hyaluronidase and ^{1}H NMR was conducted. As the result, two ^{1}H-NMR peak at the 1.3 and 1.6 ppm which represents the peak of alkyl chain of cross-linked HA was observed in the NMR spectra.

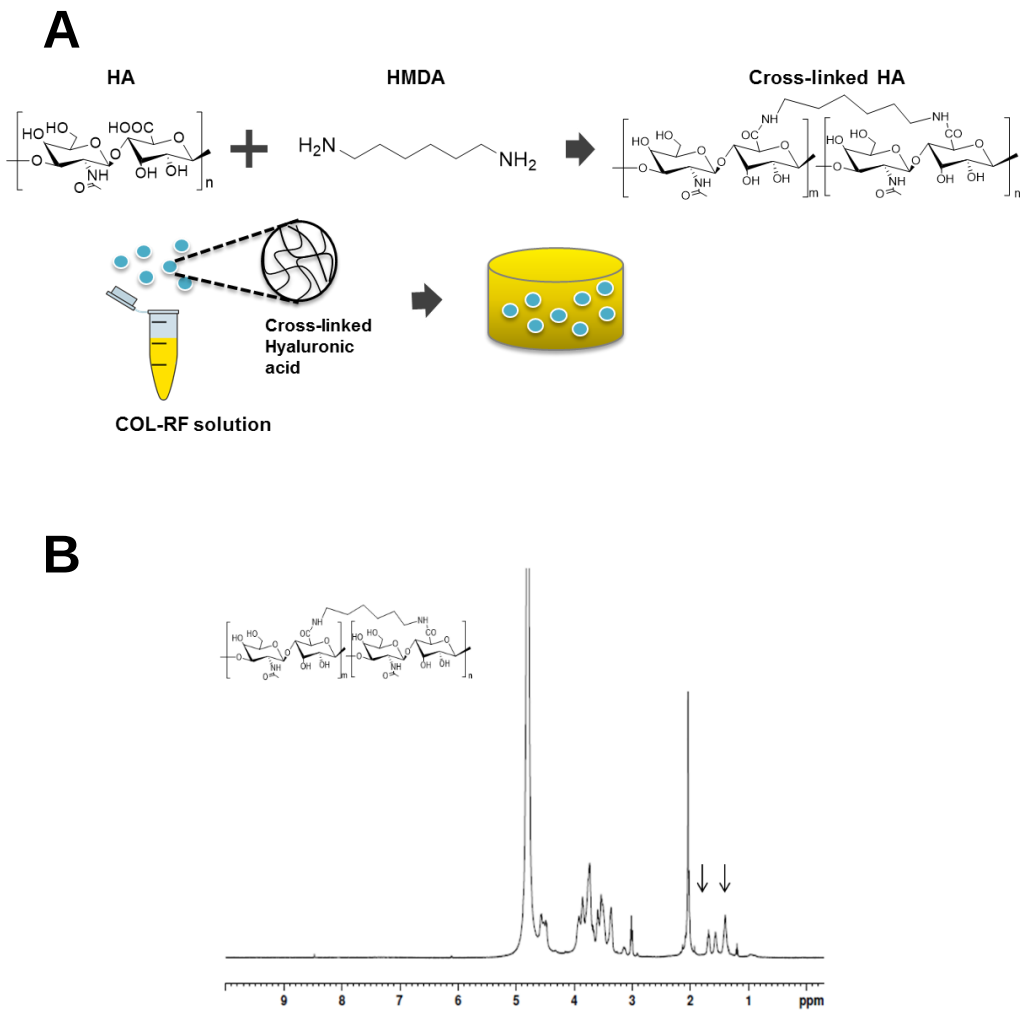


Figure 10. (A) Schematic representation of synthesis of cross-linked HA and application in collagen hydrogel. (B) ^{1}H-NMR spectra of synthesized cross-linked HA.

3.3.2 HA retention within hydrogel

To determine HA retention within construct, we conducted carbazole assay and FT-IR. First, carbazole assay was performed to confirm the release amount of cross-linked HA (XLHA) from COL-RF (Fig. 12A). Non-cross-linked HA (Non XLHA) incorporated into COL-RF was used as a control. Initial amount of HA were 1.5 mg/construct and each samples were immersed in the distilled water for 6 days. After 6 days, distilled water was collected and then released amount of HA were analyzed by carbazole. As the results, XLHA and Non XLHA exhibited the mass percentage of released HA was 4.49 and 31.41% respectively.

Furthermore, we performed FT-IR measurements for structural analysis of COL-RF-HA to identify HA existence within hydrogel after 7 days (Fig. 12B). XLHA and COL-RF were analyzed for comparison. In the COL-RF-HA spectrum, the peaks at 1252 and 1408 cm^{-1} can be attributed to the glucuronic acid and symmetric C-O stretching modes of the planar carboxyl groups in the hyaluronic acid as previously reported[32].

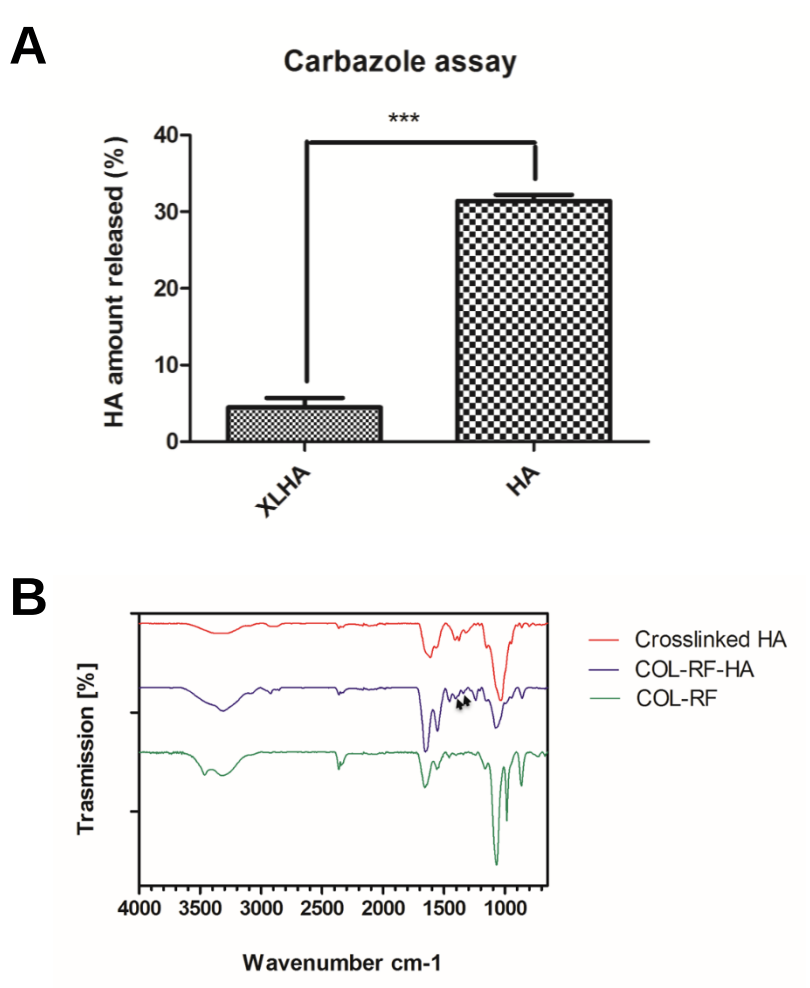


Figure 11. (A) Carbzole assay for confirming the release amount of cross-linked HA from hydrogel. Non-cross-linked HA was used as the control. (B) FT-IR spectra of cross-linked HA, photo-cross-linked collagen hydrogel with cross-linked HA or without HA.

3.3.3 Gene expression analysis of fibrochondrocytes in collagen hydrogels.

Real-time PCR was performed for 3 weeks cultured fibrochondrocytes within 3 groups of collagen hydrogels: COL, COL-RF and COL-RF-HA (Fig. 13). To assess the whether fibrochondrocytes in each hydrogels can promote ECM production, expression of type I collagen, type II collagen and aggrecan was analyzed. COL-RF-HA group showed increase of the gross gene expression level compared to other two groups. Above all, in case of type I collagen expression that was slightly decreased in COL-RF, there was significant increase exists in COL-RF-HA. Also, aggrecan expression were significantly elevated in the COL-RF-HA compared to COL and COL-RF.

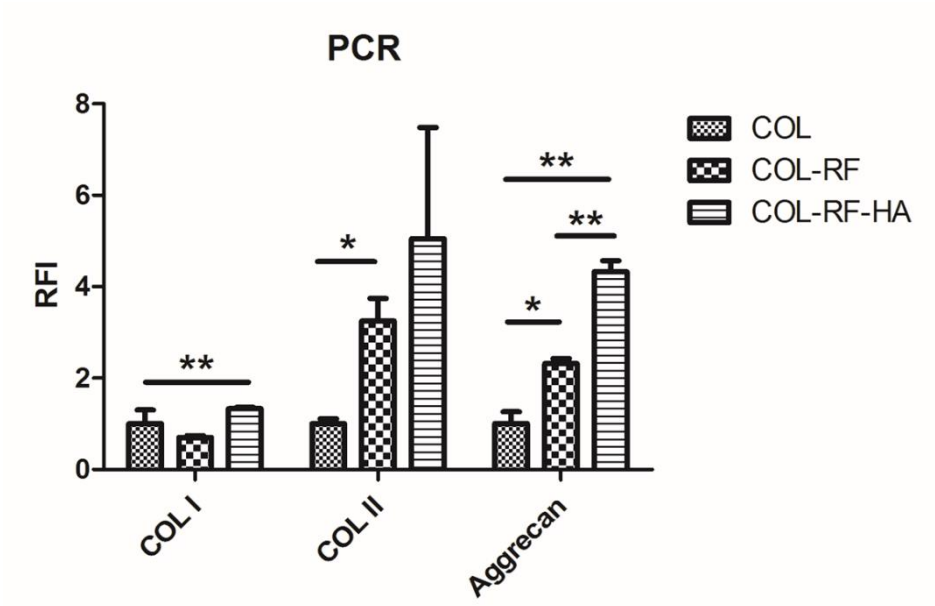


Figure 12. Relative gene expression level for the meniscus related factors, collagen I, collagen II, aggrecan after 3 weeks of rabbit fibrochondrocyte culture within each construct. Gene expression was normalized by collagen hydrogel of 1 day culture. Error bars represent the standard deviation on the mean for n=3.

3.3.4 In vivo meniscus regeneration

After 1 week of in vitro culture, rabbit fibrochondrocytes encapsulated in COL-RF and COL-RF-HA were subcutaneously implanted into the 6 week nude mice for 4 weeks. Each hydrogels were retained its original shape over 4 weeks, however increase of mechanical property were not observed indicating immature meniscus matrix formation (Fig. 14A). We also performed histological staining on each constructs to examine the effect of COL-RF and COL-RF-HA under in vivo circumstance (Fig. 14B). It was appeared that in spite of covalent cross-link between fibrils, some part of the interior hydrogel was degraded. Interestingly, COL-RF-HA exhibited much more diffused Saf-O staining through the matrix than COL-RF which showed accumulation of the GAG just around the cell.

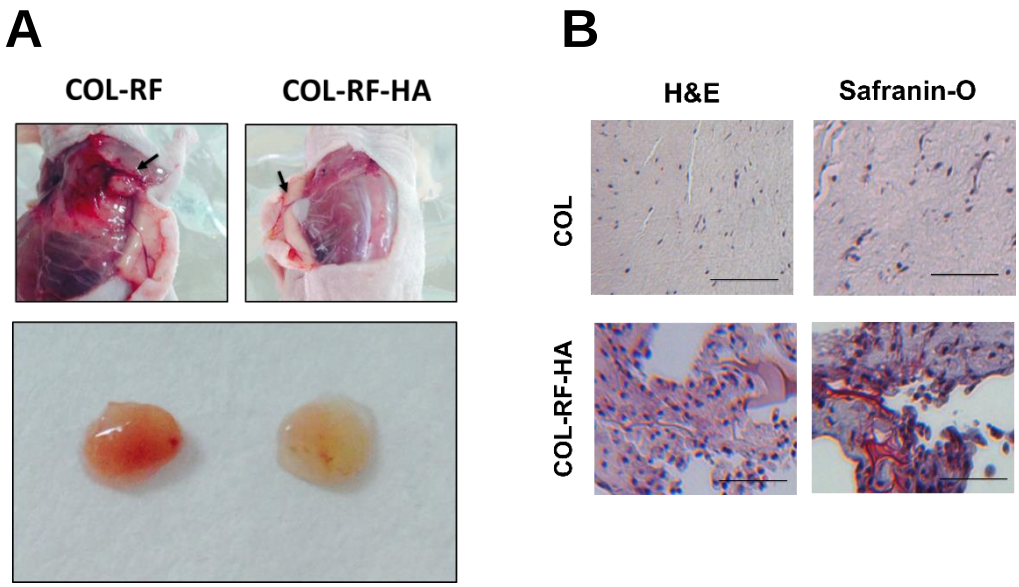


Figure 13. (A) Subcutaneous implantation of fibrochondrocyte encapsulated hydrogel groups in nude mouse for 4 weeks. (B) H&E staining and Safranin-O staining (Saf-O) of rabbit fibrochondrocyte populated hydrogel with 3 weeks culture. Scale bar=100 ㎛.

3.4 Discussion

Although COL-RF has shown improved physical property than COL that is more suitable for the meniscus scaffold, it was revealed that COL-RF does not support production of type I collagen. Previous studies on meniscus tissue engineering have shown that HA have potential for stimulating collagen remodeling, healing of the meniscal injury, and producing bizonal tissue similar to meniscus[33-35]. Hence, HA was introduced to the collagen hydrogel for complement advantageous bioactive factors for meniscus regeneration.

Because cross-linked HA is bulky and have little carboxyl group which is recognition site of hyaluronidase, cross-linked HA expected to be stay longer than non-cross-linked HA in the cell encapsulated COL-RF stimulating meniscus regeneration[36, 37]. Through carbazole assay, it was confirmed that Non XLHA could easily release from hydrogel even within 6 days. It means if this rate were maintained consistent, then there could be release of the aggregate substitute within 20 days and it is too short time to expect effectiveness on meniscus regeneration. In contrast the amount of XLHA could be retained significantly longer than COL facilitating long term HA effect on meniscus cell. Corresponding results may have been derived from bulky size of XLHA causing the interruption of mass transfer. Thus, this studies verified utility of XLHA incorporated COL-RF and it would be expected that XLHA more persistently give effect on ECM synthesis of fibrochondrocytes.

HA is already demonstrated that can promote healing of meniscus and proliferation[38]. Additionally, U Freyman et. al found that addition of hyaluronic acid induce meniscal matrix formation when cultivated in 3D culture[33]. Our findings on

gene expression analysis are consistent with this recent studies, as the fibrochondrocytes encapsulated COL-RF-HA displayed formation of native meniscus-like tissue. Therefore, the outcome of this study suggests that cross-linked HA as a small bead within collagen gel can also build up the production of meniscus extracellular matrix component similar with the addition of HA in the culture medium. In particular, we found out the supplement of cross-linked HA could stimulate the production of proteoglycans in vivo compared to COL-RF. It is probably due to COL-RF-HA produced GAG more rapidly than other group as the previously studied on HA effect of GAG synthesis. Thus, extensive ECM matrix might be formed before degradation in the COL-RF-HA. While COL-RF showed GAG deposition with surrounding cells results from less activity on GAG production. However, in vitro histological analysis of COL-RF were different from this results displaying extensive GAG deposition of pericellular region and matrix. The reason for this discrepancies could be the rapid degradation rate in the in vivo environment. Since collagen hydrogel is susceptible to degrade in vivo environment due to presence of collagenase, there could be no sufficient time to broaden GAG deposition through the matrix. Based on these findings, we therefore suggest that addition of COL-RF with cross-linked HA could enhance the fibrochondrocytes function of ECM synthesis.

One limitation with this study is that its mechanical property is still substantially lower than real meniscus tissue. However, we expected that the external stress adapted to the hydrogel would not be larger as their shape get fit to meniscus defect through injection. Thus, further study is required to prove hydrogel could survive from living body environment.

Chapter 4. Conclusions

For potential meniscus regeneration, we obtained collagen hydrogel scaffold characterized by delayed degradation, less contractility, and improved mechanical property through riboflavin induced photo-cross-linking. Furthermore the results of gene expression analysis and histology suggests that fibrochondrocytes may induce synthesis of GAG after 3 weeks culture within photo-cross-linked collagen gel. To activate fibrochondrocytes function of meniscal ECM molecule synthesis such as type I collagen, we supplement cross-linked HA to scaffold and it was shown to be achieved the desired ECM production according to in vitro gene expression analysis and in vivo histological evaluation. These results indicated that physically improved injectable collagen hydrogel and bioactive HA supplement might be beneficial for scaffold-based meniscus regeneration.

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국 문 초 록

연골판 결손은 흔하게 발생하는 무릎 질환 중 하나이지만 이를 완전히 치유할 수 있는 기술은 아직까지 개발된 바 없다. 현재 진행되고 있는 연골판 재생 연구 중 각광받고 있는 것은 지지체를 이용하여 세포 기반의 조직 재생을 유도하는 방법이다. 특히 연골판 기질의 대부분을 차지하고 있는 콜라겐 I은 하이드로겔 과 같은 지지체의 재료로 널리 사용되고 있다. 그러나 자연적으로 발생하는 콜라겐 하이드로겔의 경우 매우 낮은 강도와 빠르게 분해되는 성질을 가지기 때문에 임상적으로 다루기가 힘들다는 단점을 지닌다. 따라서 본 연구의 목적은 라이보플라빈을 광개시제로 사용하여 콜라겐 피브릴 사이의 공유결합을 유도하고 이를 통하여 한층 강화된 물리적 성질을 가지며 보다 연골판 지지체에 적합한 콜라겐 하이드로겔을 만드는데 있다. 본 실험에서는 라이보플라빈의 농도와 자외선 노출 시간을 최적화하여 강도가 증가하고 효소에 의한 분해가 느려진 하이드로겔을 제작하였다. 또한 겔 내에 연골판 세포를 3 주 동안 배양한 결과 광가교결합된 콜라겐 하이드로겔에서 대조군에 비해 콜라겐 Ⅱ와 aggrecan의 합성에 관여하는 유전자 발현이 증가한 것으로 나타났으며 이를 조직학 염색을 통해 확인했다. 더 나아가 연골판 재생을 촉진한다고 알려진 히알루론산을 광가교결합된 하이드로겔에 추가적으로 첨가한 결과 콜라겐 Ⅱ, aggrecan 뿐만 아니라 콜라겐 I의 유전자 발현이 대조군에 비해 증가함을 확인하였다. 따라서 본 연구는 기존 콜라겐 하이드로겔의 단점을 극복하여 조직 재생에 활용 가능한 세포기반의 하이드로겔을 만들고 여기에 생리활성 물질을 첨가하여 연골판 기질의 합성을 유도했다는 점에 의의가 있다.

주요어 : 콜라겐, 하이드로겔, 라이보플라빈, 하이알루론산, 연골판 조직공학, 연골판세포

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