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Characterization of Collagen Type I and II Blended Hydrogels for Articular Cartilage Tissue Engineering

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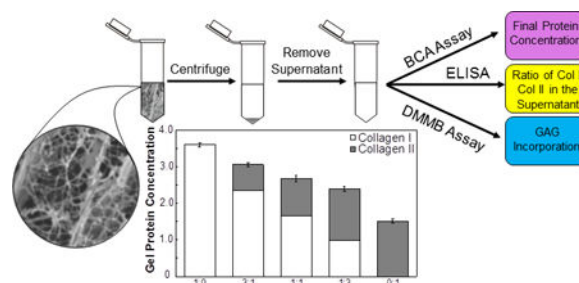
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Abstract

Biomaterials that provide signals present in the native extracellular matrix have been proposed as scaffolds to support improved cartilage regeneration. This study harnesses the biological activity of collagen type II and the superior mechanical properties of collagen type I by characterizing gels made of collagen type I and II blends. The collagen blend hydrogels were able to incorporate both types of collagen and retained chondroitin sulfate and hyaluronic acid. Cryo-scanning electron microscopy images showed that the 3:1 ratio of collagen type I to type II gels had a lower void space percentage (36.4%) than the 1:1 gels (46.5%). The complex modulus was larger for the 3:1 gels ($G^*=5.0$ Pa) compared to the 1:1 gels ($G^*=1.2$ Pa). The 3:1 blend consistently formed gels with superior mechanical properties compared to the other blends and has the potential to be implemented as a scaffold for articular cartilage engineering.

Graphical Abstract



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SUPPORTING INFORMATION

Fluorescent images of collagen type II immunostaining in hydrogels of different ratio blends (Figure S1). Graph of the average fibril diameter in gels at different ratio blends (Figure S2). CryoSEM images and quantitative analysis of collagen networks in 3:1 collagen type I to collagen type II blends with no GAGs or supplemented with CS, HA, or both CS and HA (Figure S3). Graph that compares the complex moduli of gels prepared with different collagen type I to collagen type II blends (Figure S4). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

collagen hydrogel; chondroitin sulfate; ELISA; rheology; cryoSEM; extracellular matrix; fibrillogenesis; osteoarthritis; hyaluronic acid

INTRODUCTION

Osteoarthritis (OA) is a debilitating condition that affects over 27 million Americans and is defined by degradation in articular cartilage extracellular matrix (ECM).¹ Patients suffer from pain and stiffness in the joints associated with the onset of OA. Tissue that is damaged by OA is a major health concern since cartilage tissue has a limited ability to self-repair due to the lack of vasculature in cartilage and low cell content.²

Tissue engineering seeks to repair damaged cartilage by introducing an optimized combination of cells, scaffold, and bioactive factors that can be transplanted into a patient.³ To prevent rejection and to provide a favorable environment for the attachment of cells, it is important to ensure that the biomaterial used for the scaffold is biocompatible. In addition, scaffold properties can induce the differentiation of encapsulated cells to a specific lineage.³ Finally, the mechanical properties of the scaffold must match those of the surrounding tissue.⁴

Many different biomaterial scaffolds are being studied as a way to repair damaged articular cartilage due to OA.⁵ For example, hydrogel scaffolds made of collagen promote the formation of cartilage by encapsulating cells and mimicking native tissue.⁶ The conservation of the structure and sequence of collagen across species has facilitated the biomedical application of collagen from many different sources.⁷ Collagen type I hydrogels with embedded mesenchymal stem cells (MSCs) promote differentiation to chondrocytes and repair cartilage defects.⁶ However, it has been shown that collagen type II hydrogels promote the differentiation of embedded MSCs to chondrocytes more efficiently than collagen type I gels.^{8,9} Collagen type II stimulates a more rounded cell shape, which is an important determinant for stem cell differentiation.¹⁰ In addition, collagen type II and alginate hydrogels have been shown to initiate and maintain the differentiation of MSCs to chondrocytes without the addition of other bioactive molecules.⁹

Collagen type II makes up 90–95% of the collagen produced by chondrocytes in the ECM and is a promising scaffold material for use in articular cartilage.¹¹ However, when compared to collagen type I, collagen type II is glycosylated to a greater extent.^{12,13} The high number of bulky disaccharide groups in collagen type II appears to hinder the formation of highly ordered fibrils.^{12,13} Without cross-linking, collagen type II exhibits poor mechanical properties when forming a hydrogel on its own.¹⁴ Previous studies have blended collagen types to change the mechanical properties of collagen hydrogels.¹⁵

During fibrillogenesis, collagen organization is affected by pH, ionic strength, and interactions with other components in the matrix. Previously, our lab created and characterized hydrogels made from collagen type I and III blends for use in skin and vasculature tissue engineering, and the addition of collagen type III increased the rate of

collagen fibrillogenesis.¹⁵ Many different studies have investigated the effects of glycosaminoglycans (GAGs), an important non-fibrillar component of the native cartilage tissue ECM, on the rate of fibrillogenesis and collagen fibril diameter, and the conflicting data is summarized in the referenced paper.¹⁶ GAGs attach to a protein core to form proteoglycans, which comprise 4–7% of the wet weight of healthy cartilage, and allow articular cartilage to withstand compressive forces.^{17,18} Creating a collagen blend hydrogel with GAGs would thus more closely mimic the native structure of articular cartilage.¹⁹ In this study, we sought to examine the effects of GAGs on collagen fibrillogenesis and to determine the amount of GAGs incorporated into the collagen hydrogel. Thus, we are interested in the interactions between a blend of collagen type I, collagen type II, and GAGs such as chondroitin sulfate (CS) and hyaluronic acid (HA).

The objective of this study was to create a hydrogel with a blend of collagen types I and II, and it was hypothesized that these blended hydrogels would have superior mechanical properties compared to gels made with collagen type II alone. We investigated whether the addition of collagen type I to a collagen type II hydrogel altered the amount of protein incorporated into the gels, the gel's mechanical properties, and the structure of the collagen network. In addition, we examined the effects of adding HA and/or CS on gel formation.

MATERIALS AND METHODS

Gel Preparation

Collagen type I, which was extracted from rat tail, was purchased from BD Biosciences (Franklin Lakes, NJ). Lyophilized chicken sternal collagen type II and hyaluronic acid ($1.5 - 1.8 \times 10^6$ Da) were purchased from Sigma-Aldrich (Saint Louis, MO). Sodium chondroitin sulfate from shark cartilage was purchased from Seikagaku (Tokyo, Japan). Stock solutions of both collagen types I and II were prepared in 20 mM acetic acid at a concentration of 5 mg/mL. The pH values of the solutions were raised to 7.4 with the addition of 10x phosphate buffered saline (PBS), 1 M NaOH, and 1x PBS, and the final concentrations of the collagen solutions were 4 mg/mL. The gels were prepared with collagen type I: collagen type II ratios of 1:0, 3:1, 1:1, 1:3, and 0:1 (Table 1). Glycosaminoglycans (e.g., CS or HA) were dissolved in 1x PBS to a concentration of 10 mg/mL and were added prior to polymerization. Gels were made with CS, HA, or a 1:1 ratio of CS to HA at a final concentration of 0.2 mg/mL of added glycosaminoglycans.

Collagen Incorporation into a Gel

The amount of collagen incorporated into the gels was determined using a previously described method.^{15,20} Briefly, the samples were heated at 37°C overnight to ensure that the solutions had polymerized. The gel was centrifuged for 15 minutes at 11000g. The supernatant was separated from the pellet by decanting. The supernatant from each sample was tested ($n = 3$) for the total amount of collagen present using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) following the manufacturer's protocol. Standard curves with different concentrations for each collagen type I to collagen type II ratio (1:0, 3:1, 1:1, 1:3, and 0:1) were used to determine the amount of collagen present within the supernatant. The

amount of protein in the gel was calculated indirectly as the total amount of collagen prepared minus the amount of collagen in the supernatant.

A modified enzyme-linked immunosorbent assay (ELISA) was then performed to measure the amount of collagen type II in the supernatant ($n = 3$). A standard curve was created by adsorbing collagen with different ratios of collagen types I and II on a 96-well plate with a high binding surface (Corning, Corning, NY). The supernatant from the hydrogels and different ratio blends of collagen type I and II were diluted to a total protein concentration of 10 $\mu\text{g/mL}$. All samples and standards were then diluted 1:100 in 1x PBS and adsorbed onto the surface of a 96-well plate for 24 hours at 4°C. The wells were rinsed three times with blocking buffer (5% non-fat powdered milk in 1x PBS), blocked with blocking buffer for 2 hours at room temperature, and rinsed three more times with blocking buffer. The plate was incubated for 24 hours at 4°C with a primary antibody for collagen type II (Abcam 34712, Cambridge, MA). The wells were rinsed with blocking buffer before being incubated with an HRP-conjugated secondary antibody (Life Technologies 16035, Carlsbad, CA) for 2 hours at room temperature. A substrate reagent solution from a Substrate Reagent Pack (R&D Systems, Minneapolis, MN) was added to each well and incubated for 20 minutes at room temperature. A stopping solution of 2N H_2SO_4 was added to each well, and the absorbance was determined on a Spectromax M5 plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Using the standard curves with the different collagen ratios, the ratios of collagen type I to collagen type II in our samples were quantified. The amounts of collagen types I and II in the gels were calculated indirectly by using the measured total protein concentration in the supernatant and the amounts of collagen types I and II in the supernatant.

CS and HA Incorporation into a Gel

A dimethylmethylene blue assay (DMMB) was performed to measure the amount of CS in the supernatant from gels made with varied amounts of HA or CS ($n = 3$). Specifically, 20 μL of the supernatant were mixed with 180 μL of DMMB reagent. The absorbance was measured on a plate reader at 525 nm. A standard curve of CS was created to quantify the amount of CS in the supernatants. The amount of CS retained in the gel was calculated indirectly.

A hyaluronic acid sandwich ELISA assay (Echelon Biosciences, Salt Lake City, UT) was performed following the manufacturer's protocol. Samples and standards were prepared in 1x assay buffer, and 100 μL was incubated in each well of the HA detection plate for 1 hour. The plate was washed three times with 1x Tris Buffered Saline (TBS) before 100 μL of HA detector was incubated for 1 hour. After three more washes with 1x TBS, 100 μL of 3,3', 5,5'-Tetramethylbenzidine (TMB) solution was added and allowed to develop for 20 minutes. Finally, 50 μL of 1 N H_2SO_4 was added to stop the reaction before absorbance was read at 450 nm.

Cryo-Scanning Electron Microscopy (cryoSEM)

Samples for cryoSEM were prepared as previously described.¹³ Briefly, collagen gels ($n = 2 - 4$) with different ratios of collagen type I to II were prepared on SEM holders and were

incubated overnight at 37°C to allow polymerization of the proteins. Samples with a 3:1 ratio of collagen type I to collagen type II with added CS, HA, or a 1:1 ratio of CS to HA were also prepared for cryoSEM imaging. The sample holders were then moved to the cryo holder, frozen in liquid nitrogen slush, moved to a Gatan Alto 2500 prechamber (Gatan Inc., Pleasanton, CA), cooled to -155°C, and fractured. The samples were sublimated at -90°C for 10–15 min and sputter coated for 120 s with platinum. Afterwards, the samples were transferred to the microscope cryo-stage, which had been cooled to -145°C, for imaging. All samples were imaged with an FEI NOVA nanoSEM field emission SEM (FEI, Hillsboro, OR) using the TLD (through the lens) or ET (Everhart-Thornley) detector operating at 5 kV accelerating voltage.

Fibril diameter measurements ($n = 782$) were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). As previously described, a perpendicular line was drawn across a fibril to obtain a measurement.¹⁵ Three blinded individuals took 10 fibril diameter measurements per image, and each individual analyzed three or more images of each type of gel.

ImageJ software was also used to obtain void space information ($n = 15$) from the cryoSEM images. A Diameter J plugin was used to segment the images and determine the fraction of image containing void space.

Rheology

An ARG2 rheometer (TA Instruments, New Castle, DE) was used to perform rheological analysis with a 20-mm cone geometry. Gels ($n = 4$) were subjected to frequency sweeps from 0.01 to 1 Hz using a controlled stress of 0.5 Pa.

Statistics

The data are represented as a mean with error bars corresponding to one standard deviation. Single factor analysis of variance (ANOVA) and Tukey's *post hoc* tests were performed for the total protein concentration in the gels, the percentage of CS retained, and rheology data. Nested factorial models were used to perform ANOVA and Tukey's *post hoc* tests to analyze the fibril diameter and void space percentage data. For all statistical tests, a value of $\alpha = 0.05$ was chosen, and significance was chosen to be a p-value at or below 0.05.

RESULTS AND DISCUSSION

The total amount of protein in the supernatant was measured using a BCA assay and was used to calculate the total amount of protein in the gel. As the ratio of collagen type I to collagen type II decreased and thus the amount of collagen type II used to create the hydrogel increased, there was a statistical decrease in the final protein concentrations in the gels (Figure 1A). An ELISA was used to measure the amount of collagen type II in the supernatant, and this information was used to calculate the amounts of collagens type I and II in both the supernatant and gel. As the ratio of collagen type I to collagen type II in the starting solution decreased, the amounts of collagen types I and II incorporated in the gel respectively decreased and increased (Figure 1B and 1C). The amount of collagen type I in the gel decreased proportionally as the ratio of collagen type I to collagen type II decreased.

However, the amount of collagen type II was not inversely proportional to the ratio of collagen type I to collagen type II. Instead, there was no statistically significant difference in the amount of collagen type II incorporated in the gel when the ratio of collagen type I to collagen type II was decreased from 1:3 to 0:1. A subset of the gels were immunostained for collagen type II to verify collagen type II incorporation in the gel (Figure S1). As expected, when more collagen type II was incorporated in the gel, an increase in fluorescence signal was observed. Since the amount of collagen type II incorporated in 0:1 gels did not differ from the 1:3 gels, the 0:1 gels were no longer considered in future experiments.

Next, we investigated whether the final protein concentrations in the blended collagen gels were altered due to the addition of HA and/or CS. The final protein concentration in the supernatant was measured for three different ratios of collagen type I to collagen type II (3:1, 1:1, and 1:3). Gels had no HA or CS added or were supplemented with HA, CS, or a combination of both HA and CS. When only HA or only CS was included, there was a significant increase in the total amount of protein incorporated into the gel. Thus, the addition of HA and/or CS did not negatively impact the total concentration of protein in the 3:1, 1:1, or 1:3 gels and therefore did not inhibit gel formation (Figure 2). The increase of protein incorporated into the gels upon addition of only CS is consistent with experiments performed by Stuart *et al.* using collagen type I gels.¹⁶ Stuart and coworkers found that adding CS decreased the amount of collagen in the supernatant and thus resulted in more collagen incorporated into the gel.¹⁶ In previous experiments, CS also increased the rate of fibrillogenesis of collagen type I and resulted in fibrils of smaller diameter.²¹ These results also indicated that the addition of CS increased the number and shape of nucleation sites and promoted the aggregation of collagen molecules end-to-end.²²

The percentage of CS retained in the gel was calculated using the supernatants from the gels supplemented with CS or both HA and CS (Figure 3A). There was a statistically significant difference in the percentage of CS retained when adding only CS to gels made with different ratios of collagen type I to II. In addition, there was a statistical difference in the percentage of CS retained when adding a 1:1 ratio of CS to HA to the different gel blends tested (3:1, 1:1, and 1:3). The interactions between CS and collagen are known to be ionic since increasing ionic strength reduces the binding of CS.^{23,24} Furthermore, collagen type II is believed to bind more CS than collagen type I due to a stronger ionic interaction between collagen type II and GAGs. For example, Pieper *et al.* attempted unsuccessfully to remove GAGs from bovine tracheal cartilage collagen type II by washing with a high ionic strength solution.²⁵ In this study, the amount of collagen type II in the supernatant significantly increased with a decrease in the ratio of collagen I to collagen II (Figure 1), and these results could explain the significant decrease in the percentage of CS retained within the gel. When comparing the percentage of CS retained for gels with only CS added to gels with CS and HA added, there was no statistical difference for the 3:1 gels, but there were statistical differences for the 1:1 and 1:3 gels.

The percentage of HA retained in the gel was calculated using the supernatants from the gels supplemented with HA or both HA and CS (Figure 3B). There was no significant difference between the three different hydrogel blends when HA was added. When HA and CS were added, there was also no significant difference between the three different ratios. There were

statistical differences between gels that had HA added and gels that had both CS and HA added for the 3:1 and 1:3 gels. However, there was no statistical difference between the 1:1 gels that had HA compared to 1:1 gels that had CS and HA. The HA that was used for these experiments was high molecular weight HA ($1.5 - 1.8 \times 10^6$ Da). High molecular weight HA chains form topological interactions between chains that reduce their mobility.²⁶ Collagen molecules are separated from the HA molecules due to the reduction in mobility and topological hindrance. The separated collagen molecules then begin to nucleate and aggregate to form fibrils without interacting with the HA molecules. In addition, HA is known to form aggregates with ECM molecules. The aggregates of ECM molecules form a viscous barrier that inhibits the displacement of macromolecules in chondrocyte cultures.²⁷ These previous findings suggest that, in our experiments, supplemented HA interacts with the added CS and precludes the CS from being incorporated within the collagen gels. The 1:3 blend was not pursued in further experiments due to the significant decrease in CS incorporated into the gels when compared to the 3:1 and 1:1 gels.

CryoSEM was performed to observe the network and structure of the collagen fibrils in the gels. Figure 4A shows representative cryoSEM images of different ratio blends of collagen type I to collagen type II. In these images, collagen fibrils and the networks these fibrils form within the gels can be seen. Furthermore, qualitatively, the fibril diameters appear to be similar between the gels, but the void space seems to increase with the addition of collagen type II. Thick lamellar-like structures are also present in the SEM images, and similar structures have been observed in other SEM images of collagen hydrogels.¹⁵

To quantify our observations, the collagen fibril diameters were measured using ImageJ software, and the diameter distribution in the gels at different ratio blends is presented in Figure 4B. The 1:0, 3:1, and 1:1 gels showed unimodal distributions, and the average fibril diameter showed no significant differences among the different blends (Figure S2). ImageJ, along with a DiameterJ plugin, was used to obtain the void space percentage in the gels (Figure 4C). The 1:1 gels showed a significant increase in void space percentage (46.5%) compared to gels with ratios of 1:0 (35.1%) and 3:1 (36.4%).

CryoSEM images of 3:1 collagen type I to collagen type II gels with no GAGs or supplemented with CS, HA, or both CS and HA were also analyzed to examine the collagen fibril network within the gels (Figure S3A). The collagen fibril diameters (Figure S3B) and void space percentages (Figure S3C) were measured using ImageJ software, and there were no significant differences observed.

The unimodal distribution shape of fibrils (Figure 4B) obtained in this study is similar to what was previously shown for gels composed of collagen type I only and collagen type I and collagen type III blends.^{15,16,28,29} However, gels composed of only collagen type I have also shown bimodal distributions.^{15,16,30} The fibril diameter ranges exhibited by collagen type I only gels (up to 0.65 μm) and collagen type I and II blends (up to 0.6 μm) were larger than those previously reported for gels composed of collagen type I only (up to 0.4 μm)^{16,29} and collagen type I and III blends (up to 0.2 μm).¹⁵ The addition of collagen type II to the blends did not significantly affect the average fibril diameter with or without the addition of GAGs (Figures S2 and S3B).

The void space percentage results obtained for the gels without GAGs (Figure 4C) are similar to previous studies that showed gels composed of 70% collagen type I and 30% collagen type III or gels composed of 30% collagen type I and 70% collagen type III had an increase in void space percentage (~45–50%) compared to gels composed of collagen type I only (30%).¹⁵ The significant increase in void space percentage as the ratio of collagen type I to collagen type II in the blends decreased suggests that the collagen type II addition limits fibril formation. Collagen type II may coat the fibrils formed by collagen type I and inhibit lateral aggregation of fibrils as has been shown for collagen type I and III gels³¹. This interaction between collagen type I and II could affect the stability of the gel and, thus, its mechanical properties.

Another explanation for the results seen from the analysis of the SEM images could be due to the fact that addition of collagen type II reduced the total collagen concentration in the gel (Figure 1A) and also reduced the collagen type I concentration (Figure 1B). A previous study showed that hydrogels have statistically similar fibril diameters when polymerized with varying collagen type I concentrations,³² and these results are consistent with the results seen with the collagen type I and II blend hydrogels in this study. In addition, similar to the results seen for the blended collagen I and II hydrogels, another study showed that decreasing the collagen type I concentration increased the void space.³³

Frequency sweeps from 0.01 to 1 Hz were performed to characterize and understand the mechanical properties of these gels. The frequency sweeps showed that at 1 Hz, the 1:0, 3:1, and 1:1 gels respectively had average storage moduli (G') of 21.2 ± 5.9 , 4.9 ± 1.0 , and 1.2 ± 0.2 Pa (Figure 5); loss moduli (G'') of 2.4 ± 0.5 , 1.2 ± 0.1 , and 0.3 ± 0.1 Pa (Figure 5); and complex moduli (G^*) of 21.4 ± 5.9 , 5.0 ± 0.9 , and 1.2 ± 0.2 Pa (Figure S4). The addition of collagen type II to the gels significantly decreased their stiffness, and this result could be attributed to a decrease in total protein concentration in the gel (Figure 1A) and an increase in void space (Figure 4C). Previously performed studies showed concentration-dependent changes in mechanical properties.^{34,35} In particular, single collagen type I fibrils or collagen type I and agarose blend hydrogels demonstrated that higher collagen type I concentrations increased the mechanical properties.^{34,35} Other studies with collagen type I membranes,³⁶ collagen type I hydrogels,¹² and collagen type I/III blended hydrogels¹¹ correlated an increase in porosity, or void space, with a decrease in the respective mechanical properties of tensile stress;³⁶ stiffness;¹² and storage, loss, and complex moduli.¹¹

The decrease in stiffness with increasing collagen type II also suggests that the ability of collagen type II to alter network structure and fibril growth can affect the gels' mechanical properties. Previous work has shown that collagen type III also alters fibril formation of collagen I and, thus, the mechanical properties of the gel.¹⁵ However, collagen gels composed of collagen type I and III blends exhibit higher mechanical properties than the gels composed of collagen types I and II shown here. At 1 Hz, gels composed of collagen type I and III blends exhibited a storage moduli range of 115–175 Pa and a loss moduli range of 15–25 Pa. Similarly, collagen type I gels had a storage moduli of ~170 Pa and loss moduli of ~24 Pa.¹⁵ In another study, Stuart and coworkers found their collagen type I gels exhibited a storage modulus of ~145 Pa and a loss modulus of ~23 Pa.¹⁶ While these collagen type I gels exhibited higher mechanical properties than the 1:0 gels presented

herein, Shayegan *et al.* have also seen lower storage moduli (0.1 – 0.3 Pa) and loss moduli (0.7 – 1.0 Pa) for collagen type I gels at 4 mg/ml to 5 mg/ml concentrations.³⁷ The difference in mechanical properties of these gels could be attributed to different collagen type I sources, gel preparation methods, and protein concentrations. Other studies have improved the mechanical properties of their gels by incorporating HA,^{26,30,38} dermatan sulfate,²⁶ and other crosslinking agents.^{38–40} Calderon *et al.* demonstrated that noncrosslinked collagen type II gels exhibited significantly lower compressive stress (27.6 ± 6.5 kPa) and compressive modulus (1.4 ± 0.35 kPa) than those that had been crosslinked (48.9–188.1 kPa and 2.4–10.3 kPa, respectively).¹⁴ Halloran *et al.* have also presented similar results where noncrosslinked atelocollagen type II scaffolds showed lower storage moduli than gels that had been crosslinked with microbial transglutaminase.³⁸

In this study, our goal was to develop and characterize collagen blend gels that harness the biological activity of collagen type II, which is the most abundant type of collagen produced by chondrocytes and has the ability to initiate and maintain the differentiation of MSCs to chondrocytes without added bioactive molecules.^{8–11} There is a growing interest in making scaffolds for tissue engineered cartilage that utilizes collagen type II, and many current studies utilize collagen type II as a crosslinked sponge.^{41,42,43} Due to the fact that collagen type II exhibits poor mechanical properties when forming a hydrogel on its own, studies have explored ways to enhance the mechanical properties of collagen type II hydrogels by developing composite scaffolds.^{14,44,45} From the experiments conducted here, the 3:1 gels were able to incorporate both collagen type I and collagen type II within the gels. It is important to note that an antibody was able to detect collagen type II, and therefore it is likely that collagen type II would be accessible to cells embedded in the scaffolds. The 3:1 gels also retained a significantly higher amount of CS, which allows articular cartilage to withstand compressive forces, than the other blends. Adding GAGs to the collagen gels creates a more biologically relevant scaffold for articular cartilage tissue engineering. Moreover, compared to the 1:1 gels, the 3:1 gels exhibited lower void space percentages and higher storage, loss, and complex moduli. Thus, based on all these properties, the 3:1 collagen type I to collagen type II blend has the potential to be implemented as a scaffold to effectively engineer articular cartilage tissue.

CONCLUSIONS

In this study, we created hydrogels composed of different ratios of collagen type I to collagen type II to elucidate their unique properties. We demonstrated that the addition of collagen type II alters gel formation, network structure, and mechanical properties. From the five different blends we created, the 3:1 collagen type I to collagen type II ratio blend has the potential to be implemented effectively for use in tissue engineering. Future work will focus on encapsulating cells and adding growth factors within these hydrogels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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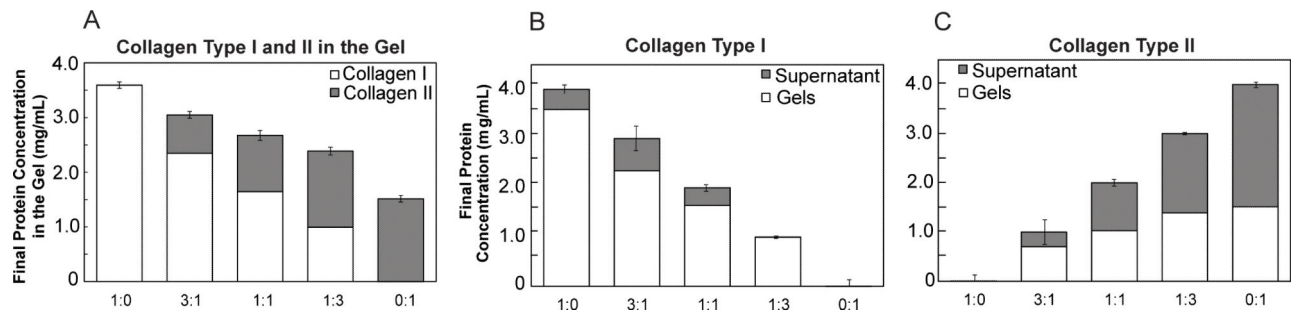


Figure 1.

(A) The final collagen concentration in the gel at different ratio blends uses white bars to represent collagen type I found in fibrillary form and gray bars to represent collagen type II measured in fibrillary form. Data ($n = 3$) are represented as the mean \pm the standard deviation of the total concentration of collagen (both collagen types I and II) in the gel. An ANOVA and Tukey's honestly significant difference *post hoc* test were performed and indicate a significant difference in the total protein concentration in the gel between each ratio ($p < 0.05$). The final concentration of collagen in the gel and supernatant for (B) collagen type I and (C) collagen type II. The white bars represent collagen content in fibrillar form, whereas the gray bars represent collagen measured in the supernatant ($n = 3$). The error bars represent the standard deviation of the amount of collagen type II in the supernatant.

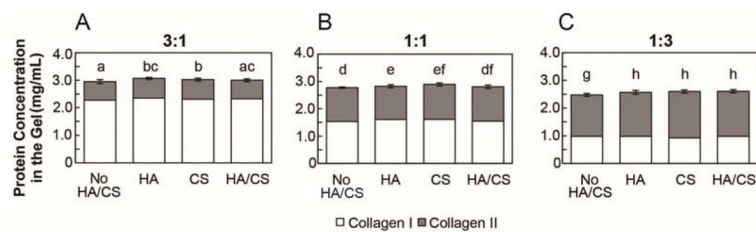


Figure 2.

The final protein concentration in the fibrils at different ratio blends of collagen type I and collagen type II with the addition of HA, CS, or both HA and CS. The gels were created containing a (A) 3:1, (B) 1:1, or (C) 1:3 ratio of collagen type I to collagen type II. An ANOVA and Tukey's honestly significant difference *post hoc* tests were performed. Different letters indicate groups with significantly different total protein concentrations incorporated in the gel ($p < 0.05$). Data ($n = 3$) are represented as the mean \pm the standard deviation.

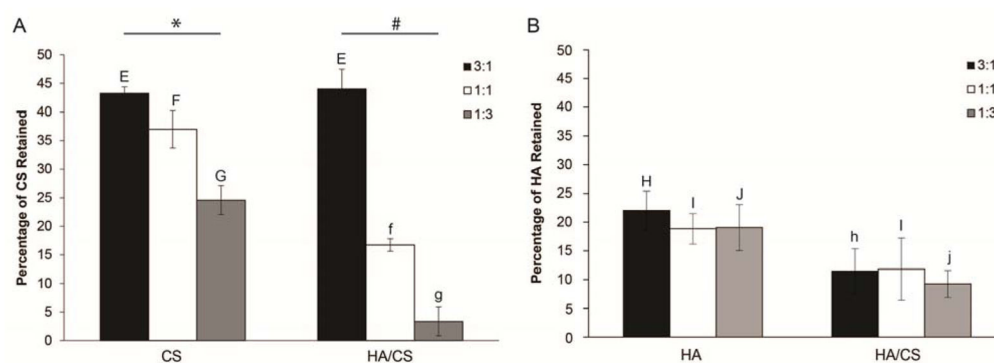


Figure 3.

The percentage of (A) CS and (B) HA retained in the fibrils. Gels were created using varying ratios of collagen type I to II (3:1, 1:1, and 1:3) with either CS, HA, or both CS and HA added into the gels. ANOVA and Tukey's honestly significant difference *post hoc* tests were performed. The * indicates significant differences ($p < 0.05$) between the three different hydrogel blends when CS is added. The # indicates significant differences ($p < 0.05$) between the three different hydrogel blends when HA and CS are added. EE indicates there is no statistical difference ($p > 0.05$) between the 3:1 gels. Ff and Gg indicate that there were statistical differences ($p < 0.05$) between the 1:1 and 1:3 gels, respectively. There is no significant difference ($p > 0.05$) between the three different hydrogel blends when either HA or HA and CS were added. Hh and Jj indicate that there were statistical differences ($p < 0.05$) between the 3:1 and 1:3 gels, respectively. II indicates there is no statistical difference ($p > 0.05$) between the 1:1 gels. Data ($n = 3$) are represented as the mean \pm the standard deviation.

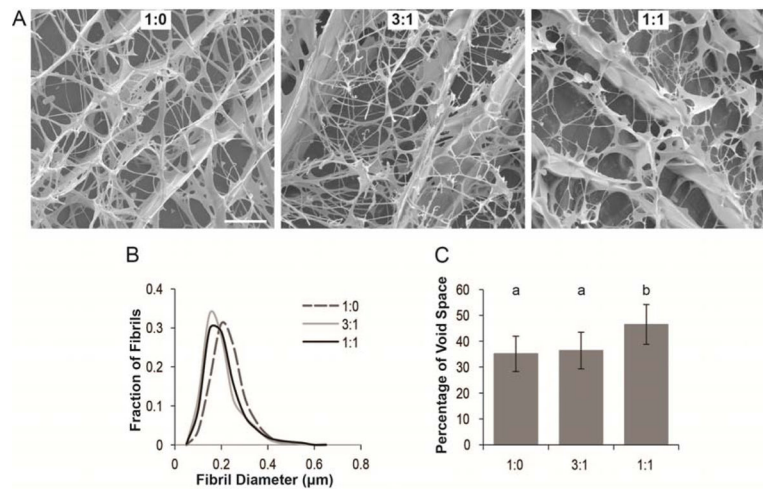


Figure 4.

Collagen networks for different ratio blends. (A) Representative cryoSEM images of different ratio blends of collagen type I to collagen type II show the collagen fibril network within the gels. Scale bar represents 5 μm. (B) Distribution of collagen fibril diameters in the gels at different ratio blends. (C) Percentage of void space for the gels based on cryoSEM images obtained at 10,000x magnification. ANOVA and Tukey's *post hoc* tests were performed on the percentage of void space data by using nested factorial models. The different letters indicate groups with a significant difference ($p < 0.05$) in the percentage of void space in the gels. Data ($n = 15$) for void space percentage are represented as the mean \pm the standard deviation.

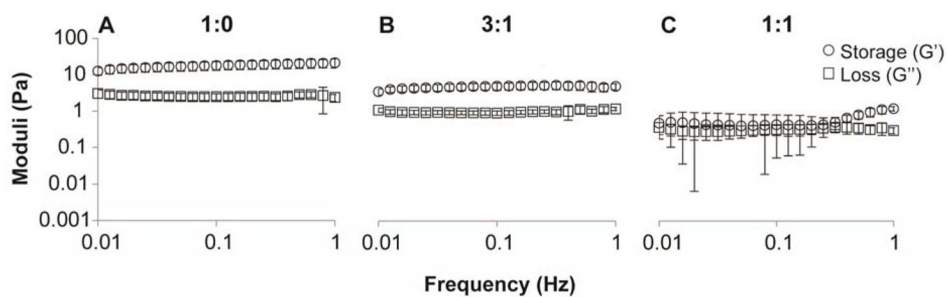


Figure 5. Frequency sweeps of storage and loss moduli of gels prepared from mixtures of (A) 1:0, (B) 3:1, and (C) 1:1 collagen type I to collagen type II. ANOVA and Tukey's *post hoc* tests were performed. At 1 Hz, a significant difference ($p < 0.05$) was observed between the storage moduli for the three different gel blends. At 1 Hz, the loss modulus for the 1:1 gels was significantly different ($p < 0.05$) from the loss moduli of the 1:0 and 3:1 gels. Data ($n = 4$) are represented as the mean \pm the standard deviation.

Table 1

Concentration of Collagen Type I and Collagen Type II in Different Ratio Blends

Ratio	Concentration (mg/mL)	
	Col I	Col II
1:0	4	0
3:1	3	1
1:1	2	2
1:3	1	3
0:1	0	4

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