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Incorporation of Types I and III Collagen in Tunable Hyaluronan Hydrogels for Vocal Fold Tissue Engineering

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Abstract

Vocal fold scarring is the fibrotic manifestation of a variety of voice disorders, and is difficult to treat. Tissue engineering therapies provide a potential strategy to regenerate the native tissue microenvironment in order to restore vocal fold functionality. However, major challenges remain in capturing the complexity of the native tissue and sustaining regeneration. We hypothesized that hydrogels with tunable viscoelastic properties that present relevant biological cues to cells might be better suited as therapeutics. Herein, we characterized the response of human vocal fold fibroblasts to four different biomimetic hydrogels: thiolated hyaluronan (HA) crosslinked with poly(ethylene glycol) diacrylate (PEGDA), HA-PEGDA with type I collagen (HA-Col I), HA-PEGDA with type III collagen (HA-Col III) and HA-PEGDA with type I and III collagen (HA-Col I-Col III). Collagen incorporation allowed for interpenetrating fibrils of collagen within the non-fibrillar HA network, which increased the mechanical properties of the hydrogels. The addition of collagen fibrils also reduced hyaluronidase degradation of HA and hydrogel swelling ratio. Fibroblasts encapsulated in the HA-Col gels adopted a spindle shaped fibroblastic morphology by day 7 and exhibited extensive cytoskeletal networks by day 21, suggesting that the incorporation of collagen was essential for cell adhesion and spreading. Cells remained viable and synthesized new DNA throughout 21 days of culture. Gene expression levels significantly differed between the cells encapsulated in the different hydrogels. Relative fold changes in gene expression of MMP1, COL1A1, fibronectin and decorin suggest higher degrees of remodeling in HA-Col I-Col III gels in comparison to HA-Col I or HA-Col III hydrogels, suggesting that the former may better serve as a natural biomimetic hydrogel for tissue engineering applications.

Graphical Abstract

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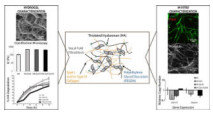

Keywords

hyaluronan; type I collagen; type III collagen; vocal folds; hydrogels; tissue engineering

1. Introduction

In mechanically active tissues like vocal folds, the cellular and extracellular matrix (ECM) organization dictate the viscoelastic properties of the tissue, which in turn, govern vibratory mechanics required for phonation.[1] Vocal fold scarring, the resulting complication of a variety of conditions such as trauma, radiation, surgery, and idiopathic causes, remains one of the most elusive laryngeal complications to treat medically and with behavioral management.[2] Tissue remodeling during natural wound healing results in excessive collagen deposition and changes in the extracellular matrix organization,[3, 4] which disrupt the viscoelastic properties and cause irreversible fibrosis and depreciation in voice quality.[5] Tissue engineering aims to use biomaterial-based scaffolds, cells and growth factors to intervene during the wound healing process and help sustain regeneration in order to restore native tissue organization.[6] Biomaterial-based scaffolds have the potential to mediate and modulate inflammation as well as proliferation of cells in the tissue, aiming to repair its native architecture.[7] In particular, biological materials from the ECM of the tissue itself are especially attractive, since they can provide the right mechanical and molecular cues for wound healing, along with being highly biocompatible.[8]

In healthy vocal folds, an organized ECM in the lamina propria maintains the viscoelastic properties required for the tissue vibrations typical in speech.[9] Major components of the ECM include collagen type I and III,[10, 11] which provide the tissue with structural support and tensile strength, elastin,[12] which provides the tissue with elasticity to sustain vibrations, and hyaluronan (HA),[13] which maintains tissue viscoelastic properties and acts as a shock absorber. Of these, HA is an attractive choice in a biomaterial/scaffold because of its polyanionic nature, bioactivity and ease of tunability.[14] In the vocal folds, HA is known to play a vital role in cell migration, differentiation, and signaling.[15] However, native HA is difficult to tune and has a low half-life in vivo, where it is degraded by enzymes.[16] Several studies have introduced functional modifications to the HA backbone in order to provide sites for crosslinking.[17–20] Of these, thiol-modified HA has been shown to be highly biocompatible for in situ encapsulation of cells.[21] Thiol modified HA can be crosslinked with the biologically neutral poly(ethylene glycol), PEGDA, to form a hydrogel that degrades slower than native HA. PEGDA is known to cause some inflammation in vocal folds[22], but used in small quantities, can enhance the residence time of the hydrogels.[23] By varying the degree of modification (thiol groups) and crosslinker (PEGDA) concentrations, HA can be tuned to match the mechanical characteristics of most soft tissues.[24] Thiolated HA-PEGDA by itself, however, provides minimal sites for cell attachment and spreading.[25]

An ideal scaffold should provide sites for attachment and spreading of cells, and guide their signaling for regeneration. Many strategies have been developed to improve cell attachment on thiolated HA scaffolds. Several studies have used thiolated HA with crosslinked gelatin to introduce sites for cell attachment through integrin binding and signaling.[21, 26] Gelatin is an unfolded version of collagen formed by denaturation of type I collagen, and does not mimic the 67nm D-periodicity of fibrillar collagen, resulting in a more disorganized network and different cellular cues in comparison to native collagen.[27] An alternative approach would be to combine thiolated HA with fibrillar collagen, but this blend has not been explored for vocal fold regeneration. Collagen type I injections increase the tensile strength of vocal fold tissue and result in better patient outcomes.[28] Type I collagen by itself or in combination with other modified forms of HA has been used in tissue engineering applications of the vocal folds [20, 29–31] due to its biocompatibility and role in regulation of cell signaling. Moreover, collagen fibrillogenesis, the spontaneous self-assembly of collagen monomers into fibrils, occurs at physiological conditions of 37°C and pH 7.4,[32] making it easy to handle.

Collagens make up 43% of total vocal fold protein.[10] Fibrillar type III collagen is co-expressed with type I in the vocal folds and constitutes more than 40% of total vocal fold collagen.[33] To date, type III collagen has not been explored as a therapeutic candidate for vocal fold regeneration. Type III collagen is upregulated during development[34] and in wound healing.[35] Furthermore, addition of type III collagen to type I collagen has been shown to regulate its fibril diameter, and results in heterotypic fibers that are more compliant[36, 37]. Therefore incorporating both, type I collagen and type III collagen, might lead to a hydrogel blend that more closely mimics the in vivo vocal fold environment.

The goal of this study was to create novel hydrogel blends with thiol-modified HA, type I collagen, and/or type III collagen blends in order to harness the mechanical stability and tunability of HA and the biological activity of the collagens in cell spreading and signaling. Thiol-modified HA was crosslinked with PEGDA to form the base hydrogel. We hypothesized that the incorporation of collagen would make the hydrogel blend more similar to native tissue architecture. Self-assembly of soluble collagen into fibrils depends on pH, temperature, ionic strength, and surfactants.[38] Electrostatic interactions between HA and collagen are also known to affect collagen fibrillogenesis.[39, 40] Collagen fibril formation is predominantly governed by hydrophobic interactions between non-polar regions of adjacent molecules. To form stable interpenetrating collagen fibrils within the highly hydrophilic nonfibrillar HA network, we reduced the net charge on the hydrogel by increasing its pH[41] in order to drive collagen fibrillogenesis at a faster rate. The resulting gel is an interpenetrating fibrillar network of collagen within a nonfibrillar porous HA matrix. The effects of different types of HA - collagen blends on hydrogel microstructure, cell viability and proliferation, and gene expression were evaluated to identify better suited candidates for vocal fold tissue engineering applications.

2. Materials and Methods

2.1 Hyaluronan Modification

Hyaluronan (HA) (MW 100 kDa, Lifecore Biomedical LLC, Chaska, MN) was thiolated according to previous protocols.[24] Briefly, HA was dissolved at 10 mg/mL in degassed Milli-Q water. Dithiobis (propanoic dihydrazide) (DTP, Frontier Scientific, Logan, UT) was added at a ratio of 2 mol DTP: 1 mol HA while the solution was stirring, and the pH was lowered to 4.75 by using 1N HCl. Next, 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC, Thermo Scientific, Waltham, MA) was added at a ratio of 0.5mol EDC: 1mol HA and the pH was maintained at 4.75 for 15 mins. Adding 1N NaOH to raise the pH to 7.0 stopped the reaction. Dithiothreitol was then added in at least 5-fold molar excess relative to the concentration of DTP to cleave the disulfide bonds and the pH was raised to 8.5 with 1N NAOH. After 24 h, the pH was lowered to 3.5 using 1N HCl and the solution was dialyzed against HCL solution (pH 3.5, 0.3 mM) with 100 mM sodium chloride (NaCl) for 2 weeks and then against HCL solution (pH 3.5, 0.3 mM) for 1 week to get rid of salts. Substitution of the glucuronate carboxyl groups by thiol groups was determined using an Ellmans assay to quantify free sulfhydryl groups. Final percent substitution of the HA was determined to be 12% and was used for all further experiments.

2.2 Hydrogel Preparation

Poly(ethylene) glycol diacrylate (PEGDA, MW 3400, Alfa Aesar, Tewksbury, MA) was used as a bifunctional electrophilic crosslinker that reacts with the substituted thiol groups on hyaluronan via Michael-type addition. The hydrogel was formed by mixing HA, PEGDA, and different formulations of collagens to form HA, HA-Col-I, HA-Col-III and HA-Col-I-Col-III hydrogels. Stock concentrations of HA and PEGDA were prepared at 3.75% and 7.18% (w/v) respectively in Dulbecco's Modified Eagles Medium (DMEM) by solubilizing them and adjusting the pH to 7.8 using 1N NaOH. The solutions were filtered through a 0.2 Syringe filter (Supor). Stock concentrations of rat-tail collagen type I (BD Biosciences, Franklin Lakes, NJ), and bovine collagen type III (MilliporeSigma, St. Louis, MO) were prepared on ice at 10 mg/mL in sterile 20 mM acetic acid. The pH of the collagen solutions was adjusted to 7.4 on ice using sterile 10× phosphate buffered saline (PBS), 1N NaOH and 1× PBS. The neutralized, ice-cold solutions of HA, PEGDA and collagen were mixed to form gels at a final concentration of 1.5% (w/v) HA, 0.718% (w/v) PEGDA, 4 mg/mL type I collagen for HA-Col I, 4mg/ml type III collagen for HA-Col III, and 2 mg/ml type I collagen + 2 mg/ml type III collagen for HA-Col I-Col III (4 mg/ml total).

2.3 Rheological Characterization

The viscoelastic mechanical properties of the gels were tested on an ARG2 rheometer (TA instruments, New Castle, DE) using 20 mm parallel plate geometry and a gap of 250–400 um. The gels were prepared on Teflon coated slides (Tekdon) and allowed to polymerize for 3 h before running frequency sweeps. The linear range of viscoelastic response was measured in triplicate with a frequency sweep from 0.1 to 100 rad/s at a controlled stress of 1 Pa.

2.4 Cryoscanning Electron Microscopy (Cryo-SEM) for Hydrogel Microstructure

Cryo-SEM was performed at the Purdue Electron Microscopy Facility to analyze the microstructure of the HA-collagen gels. Hydrogels were prepared on SEM stage holders and incubated for 2 h at 37 °C to allow polymerization. The sample holders were then moved to a cryo holder, flash frozen by immersing in liquid nitrogen and moved to a Gatan Alto 2500 prepchamber under vacuum to be fractured. Once fractured with a scalpel, the samples were sublimated at a temperature −90 °C for 10–15 mins and sputter coated with platinum. Images were taken on the FEI NOVA nanoSEM at a microscope temperature of − 140 °C using the Everhart-Thornley (ET) detector at 5kV with a spot size of 3. All samples were tested in triplicate.

2.5 Hydrogel Swelling Ratio

Hydrogels were placed in 1X PBS at 37 °C for 48 Hours. Swelling ratio (Q) was calculated as the ratio of the weight of the swollen gel to the weight of the dry gel. Swollen gels were weighed and washed 5× with distilled water and then dried under vacuum for 3 days to determine the weight of dry gels.

2.6 Degradation of HA in hydrogels

The biocompatibility of the modified HA in the HA collagen blend gels was measured using a modified carbazole assay according to previous methods.[42] HA, HA-Col I, HA-Col III and HA-Col I-Col III gels were evaluated in this study. Hydrogel blends were polymerized in four chamber slides (Nunc Lab-TekII, VWR Scientific). The slides were left at 4 °C overnight for curing. For each hydrogel blend, three replicate gels were incubated with hyaluronidase (50 units ml^{-1} in PBS) at 37 °C. At selected time points of 0, 2, 4, 6, 8, and 10 h, 750 μl of hyaluronidase enzyme solution was removed and replaced with an equal amount of fresh enzyme solution. Amount of thiolated HA degradation was measured by the release of glucuronic acid into the supernatant using a modified carbazole assay in a 96-well plate format. The absorbance of the collected solutions was measured on the M5 microplate reader at a wavelength of 550 nm. Percent HA degradation was calculated across all time points for the gels.

2.7 Cell Encapsulation in hydrogel blends

Immortalized human vocal fold fibroblasts (I-hVFFs) obtained from Dr. Susan Thibeault's lab in Madison, WI[43] were trypsinized, counted and pelleted to a final concentration of 1×10^6 cells/mL and resuspended in gel solution for embedding. 10 uL aliquots of the cell-gel suspension were pipetted onto ibidi μ-slide angiogenesis (Ibidi inc). The slides were placed in an incubator (37 °C and 5% CO_2) for 3 h to allow complete gelation, after which complete I-hVFF medium [Dulbecco's Modified Eagles Medium (DMEM, Sigma) with 10% FBS, 1% Pen/Strep, 1% minimal essential medium (MEM) non-essential amino acid solution (Sigma), and 10 mg/mL geneticin (Teknova)] was added to the wells.

2.8 Cell Viability and Cytoskeleton Organization

To visualize cell viability, the cell gel constructs were stained using the live/dead cytotoxicity kit (Invitrogen). Constructs were harvested at days 1, 7, 14, and 21 and stained

with 4 μM calcein AM and 6 μM ethidium homodimer-1 in PBS for 30 mins. Adding ethanol to one of the constructs for 45 mins was used a dead cell control. To observe actin staining, the constructs were fixed in 4% paraformaldehyde (PFA) for 30 mins, permeabilized with 0.1% Triton X-100 for 30 mins, blocked with 5% milk for 2 h, and stained with Alexa Fluor 633 Phalloidin (Invitrogen) and DAPI for 1 h. The stained constructs were imaged using a Zeiss 880 confocal microscope with a 25X oil immersion objective. Z-stacks were taken through the range of 570 μm to image the gel and the images were displayed as maximum intensity projections using ImageJ.

2.9 Assessment of new DNA synthesis in encapsulated cells

The 5-ethynyl-2′-deoxyuridine (EdU) proliferation assay was used to quantify cell proliferation in encapsulated cells according to previous methods.[44] EdU, a thymidine analogue containing a terminal alkyne group that is incorporated into newly synthesized DNA, was added to each well at a final concentration of 1 μM for 10 h for days 1, 7, 14 and 21. To analyze the amount of newly synthesized DNA, a cell-permeable fluorescent azide was conjugated to the EdU alkyne using copper-catalyzed click chemistry. Briefly, the gels were fixed with 4% PFA for 30 min, rinsed 3X with DPBS, permeabilized with 0.5% Triton-X 100 for 30 min, rinsed, blocked using 5% milk for 2 h, and incubated with Alexa Fluor 488 conjugated azide (Invitrogen) diluted in 1M Tris (pH 8.5), 25 mM copper (II) sulfate and 0.25 M ascorbic acid. DAPI was used to stain nuclei. Constructs were imaged using a Zeiss 880 confocal microscope with a 25X oil immersion objective. EdU+ cells divided by total cells counted was used to depict proliferation in cells.

2.10 Gene Expression for ECM constituents

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to analyze expression of ECM related genes for I-hVFFs embedded in the different hydrogel blends. Once harvested, the constructs were digested in hyaluronidase (7500 U/mL) for 45 min, centrifuged and lysed. RNA was extracted from the constructs using QIAshredders and RNeasy mini kit (Qiagen, Frederick, MD) using the manufacturers protocol. The purified RNA concentration was measured using a NanoDrop 2000 spectrophotometer. The RNA was then reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems) following manufacturers instructions. qPCR was performed using equal quantities (1 ng/μL) of cDNA template for 40 cycles using the TaqMan gene expression mastermix (Life Technologies, Waltham, MA), TaqMan probes for β-actin, COL1A1, COL3A1 Decorin, Fibronectin, and MMP1, and an Applied Biosystems 7500 real-time PCR machine. The cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec, and 60 °C for 1 min. Each individual sample was tested in triplicate. Data were analyzed using the linear regression of efficiency (LRE) method[45] to determine relative copy number and PCR efficiencies to show fold difference in gene expression compared to control (HA gels).

2.11 Statistical Analysis

Data are represented as means, with error bars corresponding to standard deviation. Single factor equal variance ANOVA and Tukey's post hoc tests were performed using Minitab for analysis of rheology, swelling ratio, degradation, proliferation and gene expression. A

probability value of 95% ($P < 0.05$) was used to determine statistical significance. Standard deviation of normalized gene expression was represented as uncertainty propagation using the formula $f = \frac{A}{B} \rightarrow \sigma_f = \left|\frac{A}{B}\right| \sqrt{\left(\frac{\sigma_A}{A}\right)^2 + \left(\frac{\sigma_B}{B}\right)^2}$, where A and B are uncorrelated variables with standard deviations σ_A and σ_B, and σ_f is the standard deviation of the function. [46]

3. Results

3.1 Gel Preparation

Preliminary experiments to incorporate collagen in the thiolated HA hydrogels by raising the pH of the gels between 7.2 – 7.4 were unsuccessful because the collagen precipitated out in solution once the gels were swollen. Stable incorporation of collagen in the HA-PEGDA gels was achieved by increasing the rate of fibrillogenesis by adjusting the pH to 7.8, which is close to collagen's isoelectric point.[41] Four types of HA-PEGDA hydrogel blends were evaluated in this study: HA alone, HA with type I collagen (HA-Col I), HA with type III collagen (HA-Col III), and HA with type I collagen and type III collagen (HA-Col I-Col III).

3.2 Rheological Characterization

Rheological tests were performed to examine the mechanical properties of the different gel blends. Frequency sweeps run between 0.1 – 100 rad/s showed that the hydrogels containing collagen have a higher storage modulus (G') and loss modulus (G") in comparison to HA alone (*P<0.05) (Figure 3–1). Overall, the HA hydrogel was the weakest, with a G' value of 188.5 ± 24 and a G" value of 1.2 ± 0.86 at 1 rad/s. The addition of collagen altered the mechanical properties of the gel by increasing the linear viscoelastic range, as well as significantly increasing the storage modulus of the gels, with G' values ranging from 954.9± 74.5, 881.7 ± 70.9, and 812.4 ± 175.3 at 1 rad/s for the HA-Col I, HA-Col III and HA-Col I-Col III gels, respectively. At 1 rad/s, all hydrogel blends containing collagen had significantly different G' values in comparison to the HA alone gel (*P<0.05). The addition of collagen displayed a trend toward increased loss moduli of the gels, with G" values ranging from 26.5 ± 8.59, 17 ± 3.39, and 18.1 ± 16.96 at 1 rad/s for the HA-Col I, HA-Col III, and HA-Col I-Col III gels, respectively.

3.3 Hydrogel Microstructure using Cryo-SEM

Cryo-SEM was performed to evaluate the network structure of the hydrogel blends. (Figure 3–2). For the HA alone gels, the large non-fibrillar porous structures appeared circular. Collagen within the HA can be seen as interdigitated fibrillar networks within varying pore sizes for all HA gels with collagen. A closer inspection of the images points to subtle qualitative differences between the HA-Col I, HA-Col III and HA-Col I-Col III gels. The fibrillar elements in the HA-Col III gels appeared smaller in comparison to the ones in HA-Col I or HA-Col I-Col III gels. HA-Col I-Col III gels appeared to have long interpenetrating fibrils in comparison to the short fibrils seen in HA-Col I and HA-Col III gels.

3.4 Hydrogel Swelling Ratio

To quantify the swelling ratio of the hydrogels, gels were swollen to equilibrium for 48 hours in 1X DPBS, weighed, then dried using a lyophilizer and weighed again to get their dry weight. HA hydrogels are known to swell to a large extent without dissolution of the gel. This is evident by the high swelling ratio of = 34.6 ± 3.82 for HA-only gels (Figure 3–3). Addition of collagen to the HA hydrogels significantly reduced the swelling ratio ($P < 0.05$). This is not surprising, as fibrillar collagen hydrogels are known to not swell.[47] Despite this reduction, all hydrogels have a relatively high swelling ratio, making them highly permeable, which should facilitate the exchange of oxygen, nutrients, and other water soluble metabolites.

3.5 Degradation of HA in hydrogels

Enzymatic degradation of the hydrogels was monitored to mimic in vivo conditions by incubating the gels in hyaluronidase solution. Hyaluronidase cleaves β-N-acetyl-hexosamine-(1 → 4) glycosidic bonds in HA, releasing glucuronic acid residues, which can be measured through the carbazole assay. At 10h, more than 90% of the HA in the HA gels had degraded, but only more than 60% of HA-Col I, HA-Col III, and HA-Col I-Col III gels had degraded in the presence of enzyme (Figure 3–4). In sum, thiolated HA retained its susceptibility to HAase degradation; however, the addition of both type I collagen and type III collagen, reduced the amount of HA degradation after hyaluronidase by at least 10%.

3.5 Cell Encapsulation, Viability and Cytoskeletal Reorganization

Encapsulation of cells in the hydrogels was achieved by mixing the cells with HA, PEGDA and type I and/or type III collagen followed by a two hour incubation at 37°C. The majority of cells remained viable at day 1 in all hydrogels as seen by the live/dead assay, showing that the in situ encapsulation process was biocompatible (Figure 3–5). The cells appeared to be alive and densely populated at 21 days, showing viability in 3D culture in the HA-Col blend gels throughout time of culture. However, majority of the cells in the HA alone hydrogel had migrated away from the scaffold due to lack of adhesion to the HA.

To assess cell morphology and cytoskeletal organization, the cells were stained with phalloidin, which binds to the actin network. At day 1, the cells showed a rounded morphology in all the gels (Figure 3–6). By day 7, the cells in all HA-Col hydrogels appeared to be distinctly elongated and more spindle shaped, showing organized cytoskeletal networks. The cells maintained this elongated morphology in the HA-Col hydrogels until the end of culture at day 21, pointing towards the role of collagen in providing a matrix for cellular attachment and signaling. The cell morphology and distribution appeared similar across all the different HA-Col hydrogels. Cells in the HA alone gels, however, failed to attach and spread like the ones in HA-Col gels.

3.6 New DNA synthesis in encapsulated cells

Cells maintained their ability to proliferate in the HA-Col blend gels over 21 days, as seen by the increase in cell number over time and the detection of EdU in the cells at 21 days (Figure 3–7). No proliferative cells were seen in the HA alone gels after 14 days, suggesting that the addition of collagen provided signaling cues for cell adhesion and proliferation.

3.7 Gene expression

In order to evaluate changes in fibroblast gene expression as a function of different hydrogel microenvironments, gene expression for various ECM constituents was analyzed after seven days of culture. Initially, gene expression was normalized using β-actin, a commonly used endogenous control. However, β-actin expression was not constant between the cells encapsulated in the different hydrogel blends (Figure 3–8). Therefore, we used the LRE method to quantify relative copy number of each reaction.[45, 48] COL1A1 and Fibronectin gene expression levels in HA-Col I and HA-Col III gels were significantly lower than that of HA-Col I-Col III gels (*$P < 0.05$). MMP1 gene expression was most significantly downregulated in HA-Col I hydrogels while decorin gene expression was downregulated in all hydrogels containing type I or type III collagen (*$P < 0.05$). The amplification of decorin in HA-Col III gels was later than others, which prevented an accurate determination of its expression using the LRE method; hence, it was assumed to be zero. There were no significant differences in COL3A1 gene expression.

4. Discussion

In an effort to recapitulate the native ECM environment of the vocal folds, three of the most abundant ECM molecules in the vocal fold lamina propria, namely type I collagen, type III collagen and HA were used to engineer four different hydrogel blends. Thiol-modified HA has been used for various tissue engineering applications and is known to be compatible with in situ encapsulation of cells. However, it has been widely used in combination with crosslinked gelatin instead of fibrillar collagen, which more closely replicates the vocal fold molecular composition in vivo.[21, 26] In this study, we demonstrate for the first time that the major types of fibrillar collagens found in the vocal folds, namely type I collagen and type III collagen, can be co-polymerized with thiolated HA gels. Collagen was incorporated by increasing its rate of fibrillogenesis by adjusting the pH to 7.8, which is collagen's isoelectric point.[41] This is hypothesized to drive fibrillogenesis of collagen faster than the Michael-type crosslinking between HA and PEGDA, with the ultimate result being an interpenetrating, unaligned hydrogel network. The addition of collagen into modified HA hydrogels not only alters the mechanical integrity of the gels, but also provides cues for attachment and proliferation of cells. The mechanical properties, microstructure, swelling and degradation of the hydrogels, as well as cell behavior were evaluated.

The elastic storage modulus of vocal folds lies between 100 – 1000 Pa.[1, 49] For biologically active tissue like the vocal folds, if the hydrogel is to be implanted at the site of scarring, it is important that the hydrogel characteristics closely match the in vivo tissue viscoelastic environment, as cellular responses are known to vary based on stiffness of the local physical environment.[50] Collagen hydrogels by themselves show relatively poor mechanical properties for tissue engineering purposes.[51] Using thiol-modified HA, the rheological properties can be tuned by varying the polymer weight and crosslinking density. We chose HA with a molecular weight of 100 kDa, since a higher molecular weight would result in a stiffer gel due to the added chain entanglement, and the addition of collagen already added sufficiently to the stiffness of the hydrogels (Figure 3–1). All the hydrogels are within the physiologically relevant range for mechanical characteristics, and have the

potential to be used as scaffolds for tissue engineering. Storage moduli significantly increased in the HA collagen blends, indicating that the addition of collagen modified the elastic properties of the hydrogels. HA-Col I hydrogels showed a trend towards higher G' values, which can be attributed to the shorter and thicker fibrils formed by type I collagen compared to type III collagen. Loss moduli (G") values also showed a trend towards increasing with the addition of collagen in comparison to HA-alone gels, indicating that the gels became more viscoelastic.

Cryo-SEM was used to observe the microstructure of the different gels and support the data obtained from the rheological properties of the gels. HA alone gels can be seen as a porous network formed by the covalent crosslinks within the hydrogel. Qualitatively, the pores appear to form a non-fibrillar network with interconnected channels. The addition of collagen appears to change this porous microstructure as indicated by the interpenetrating fibrillar structures present in the HA-Col blends. Various glycosaminoglycans (GAGs), including HA, are known to alter collagen fibrillogenesis.[52] Additionally, collagen fibril assembly is also dependent on the structure, assembly and concentration of GAGs.[53] The subtle differences in hydrogel microstructure due to the different types of collagens can be attributed to the chemical assembly of the HA-Col blend gels. The competing Michael-type crosslinking between thiols and acrylates increases the solution viscosity dramatically, inhibiting collagen mobility and rate of fibrillogenesis within the matrix. Increasing the pH during gelation supported more rapid collagen assembly, in part overcoming the increase in viscosity due to crosslinking. The resulting matrix formed appears to have short, interdigitated fibrils of collagen, with fibrils of type I collagen appearing thicker than fibrils of type III collagen. This is consistent with the data in the literature showing that type I collagen fibrils have a diameter of 150–300nm and type III collagen fibrils have a diameter of 25–100nm.[37]

Equilibrium swelling ratios demonstrate that the hydrogels have a high degree of swelling, and thus, can provide access to oxygen, nutrients and metabolites required by the cells in order to sustain long term culture. The addition of collagen type I reduces the swelling ratio of the gels. This observation is consistent with the literature, which demonstrates the non-swelling nature of collagen hydrogels.[47] Mehra and coworkers[53] have shown that the addition of thiolated HA to type I collagen hydrogels inhibits commonly seen collagen contraction in pure type I collagen gels, making the hydrogels more biocompatible due to inhibition of contraction. This behavior is important for long-term culturing of cells, since fibroblast-mediated contraction of collagen can provide altered signaling cues to the cells.[54] No visible contraction of gels was seen even after 21 days in culture (data not shown), despite the distinct spindle shaped elongated cytoskeletal processes seen in the HA-Col blend gels.

Another advantage of using biodegradable HA is that the degradation products of HA have been shown to be non-toxic in tissues.[55] Previous studies in our lab have shown that higher degrees of substitution (44%) reduce the biological activity of the HA.[42] While the hydrogel degradation data due to hyaluronidases cannot be explained by simple enzyme kinetics, this study demonstrated that the HA hydrogels remain bioactive despite the 12% modification on the backbone, since the enzyme can still recognize the glycosidic linkages

for cleavage. Reduced degradation in the gels containing collagen correlates with the cryo-SEM and swelling data, as it is likely that the addition of collagen fibrils within the HA pores may decrease hyaluronidase diffusion through the gels and reduce access to cleavage sites on the HA due to the reduced swelling ratio as well as the additional volume conferred by the presence of the collagen fibrils. Overall, these results demonstrate that, even following thiol substitution of the HA, bioactivity is maintained.

To determine the biocompatibility of the matrices, encapsulated fibroblasts were tested for viability until the end of culture using a live/dead assay kit. At day 1, despite the rounded morphology, most cells appeared viable, confirming that the in situ encapsulation method was biocompatible. Prolonged culture of vocal fold fibroblasts in the encapsulated gels showed that collagen is essential for viability, adhesion, and retention of cells in the matrices. Cells appeared to have migrated out of HA alone gels by day 14, owing to the lack of polar adhesion forces and proteins required for cell spreading within HA gels. Studies have shown that cells migrate faster on softer gels,[56] and that HA is a key player in cell migration,[57] which can explain this observation. On the HA-Col blend hydrogels, however, cells remained viable even at 21 days, with distinctly visible spindle shaped morphology, confirming the biomimetic nature of the blend hydrogels. Despite maintaining protein expression of the β1 integrin subunit at day 7 (supplementary figure 1), cells in HA gels remained rounded, while those in HA collagen blends were able to spread, presumably via a β1-containing integrin dimer. There is evidence in the literature that cell spreading on fibrillar collagen is mediated through α2β1 and α11β1 integrins,[58, 59] but this interaction is much weaker on gelatin, possibly due to a reduction in poly-proline-II content of gelatin.[27] Binding to gelatin is dependent on a different set of integrins (αvβ3 and α5β1),[60] which have implications on cell signaling in vivo. Crosslinking with gelatin also affects cell adhesion, with increasing non-specific non-integrin based adhesion seen due to increased gelatin crosslinking density.[27] Overall, since these studies point to reduced bioavailability of important native integrin ligands on collagen in the use of gelatin, we hypothesized that fibrillar collagens will provide superior characteristics to the hydrogels and mimic the native ECM environment better. In this study, viability did not seem to be affected during the 21 days of culture in collagen-containing gells, and the cells formed long cytoskeletal projections to interact with other cells and the ECM.

The interpenetrating fibrillar network created by collagen not only supported cell viability and adhesion, but also facilitated cell proliferation, as seen by the incorporation of EdU in proliferating cells. Differences in the proliferation rates of the cells in the different hydrogels at various time points indicate that the matrix environment affects cell proliferation. However, there is no clear trend, implying that cell proliferation is not a function of the type of collagen in the gel alone; instead, many other factors, including the overall microstructure, and the viscoelasticity of the matrix, play an important role in proliferation.

Gene expression of ECM constituents showed that the cells responded differently to the incorporation of type I collagen versus type I and type III collagen in the HA hydrogels. MMP1 is a collagenase known to breakdown interstitial type I, II and III collagens and is overexpressed during tissue repair.[61, 62] Significant downregulation of MMP1 and COL1A1 in gels containing type I collagen and type III collagen was seen in comparison to

HA alone and HA-Col I-Col III gels, suggesting lesser matrix turn over in HA-Col I and HA-Col III hydrogels as compared to the combination hydrogels. Fibronectin is a glycoprotein that plays an important role in wound healing by enhancing cytoskeletal reorganization and collagen deposition, along with acting as a chemoattractant and guiding cells to the wound healing site.[63] An upregulation in fibronectin in the HA-Col I-Col III gels could explain the higher expression of COL1A1 in those hydrogels, and taken together, relative fold changes in gene expression of these proteins suggest a higher degree of remodeling in hydrogels containing type I collagen and type III collagen together in comparison to the addition of a single type of collagen. Decorin gene expression was significantly downregulated in all hydrogels containing collagen in comparison to HA gels. Decorin binds to type I collagen and reduces its fibril diameter, thus modulating collagen fibrillogenesis,[64] and its expression during fibrosis is highly variable.[65] Downregulation of decorin has been implicated in scarless wound healing in early gestation fetal fibroblasts and fetal skin fibroblasts.[66] This effect suggests that an upregulation in decorin might be detrimental to healthy tissue composition, as disorderly collagen fibrillogenesis might result in matrix irregularities.

Overall, the addition of both, type I and type III collagen, to the hydrogels appears to support healthier tissue formation and regeneration as seen by the proliferation, viability, and gene expression data. As we hypothesized, the elevated expression of type III collagen in developmental tissues and elastic soft tissues like the vocal folds may play an important role in maintaining a physiologically relevant matrix environment.

5. Conclusion

In this study, we evaluated the biocompatibility and functionality of HA and collagen blended 3D scaffolds for in vitro static culture of vocal fold fibroblasts. Four types of hydrogels were studied, with thiolated HA-PEGDA as the base hydrogel material. By driving the rate of collagen fibrillogenesis faster, stable incorporation of fibrillar type I collagen and type III collagen in the nonfibrillar HA network was achieved. HA, HA-Col I, HA-Col III and HA-Col I-Col III hydrogels were evaluated for their mechanical properties, bioavailability and biocompatibility and their influence on vocal fold fibroblast cell behavior. All four hydrogels allowed for *in situ* I-hVFF encapsulation. The incorporation of collagen not only significantly altered the mechanical properties of the hydrogels, but also provided for cell attachment and adhesion in the blend hydrogels. The incorporation of both fibrillar type I and III collagens within the widely characterized and biocompatible thiolated HA hydrogels is an attractive candidate for tissue engineering due to its functional biomimetic nature. Cells responded differently to hydrogels containing type I collagen and type III collagen as seen by the differences in gene expression, suggesting that addition of both type I and type III collagen provides a tissue microenvironment conducive for remodeling. Future work will involve testing the regenerative and wound healing abilities of the hydrogels *in vivo*, alignment and density gradient formation of the collagen fibrils by using compression or magnetic alignment,[67, 68] as well as incorporating physiologically relevant mechanical stimulations to create in vitro functional testing models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Voice disorders affect about 1/3rd of the US population and significantly reduce quality of life. Patients with vocal fold fibrosis have few treatment options. Tissue engineering therapies provide a potential strategy to regenerate the native tissue microenvironment in order to restore vocal fold functionality. Various studies have used collagen or thiolated hyaluronan (HA) with gelatin as potential tissue engineering therapies. However, there is room for improvement in providing cells with more relevant biological cues that mimic the native tissue microenvironment and sustain regeneration. The present study introduces the use of type I collagen and type III collagen along with thiolated HA as a natural biomimetic hydrogel for vocal fold tissue engineering applications.

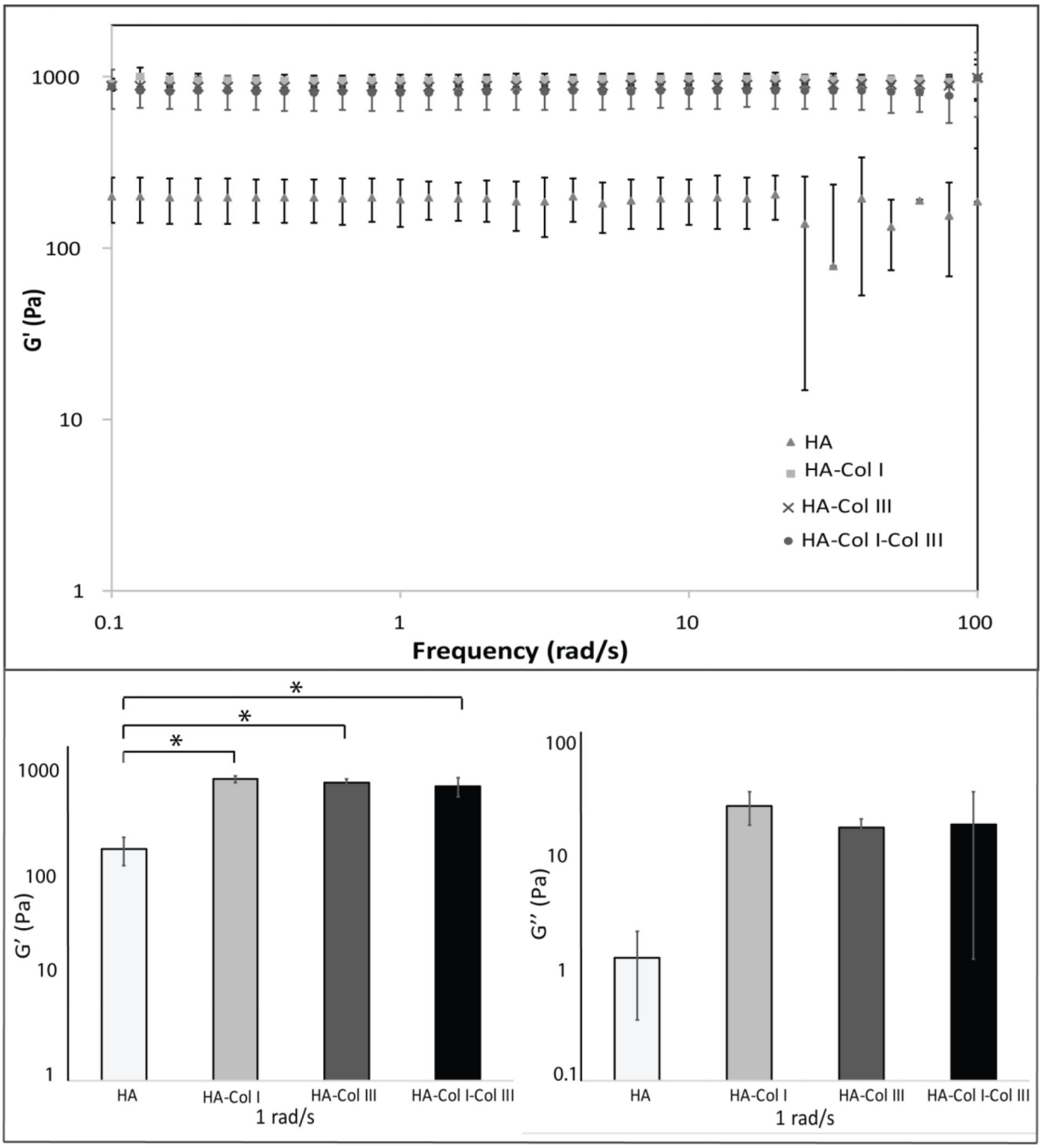

Figure 3–1. Rheological Characterization of Hydrogel Blends.

One way ANOVA was used to analyze significant differences in storage modulus (G') and loss modulus (G") of the hydrogels and indicated that the hydrogel type affected G' (*P < 0.05). Values are represented as means with error bars showing standard deviation for n=3. Tukey's post hoc test revealed that the addition of both type I collagen and type III collagen altered the mechanical properties of the hydrogels in comparison to HA alone hydrogels, with an increase in the linear viscoelastic range as well as G' values.

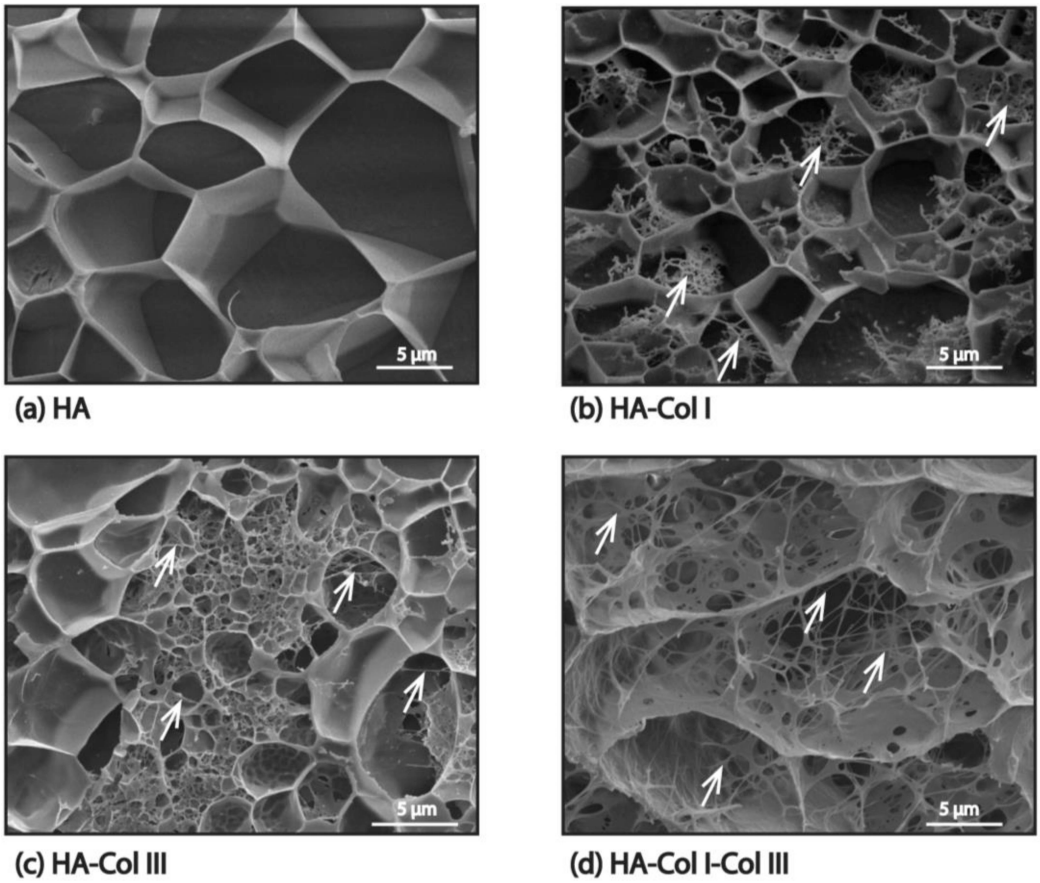

Figure 3–2. Representative Cryo-SEM Images of Hydrogel Blends

Incorporation of collagen can be seen by the interpenetrating fibrillar structures (depicted by white arrows) in the honeycomb-like, non-fibrillar HA. Scale bar = 5 μm.

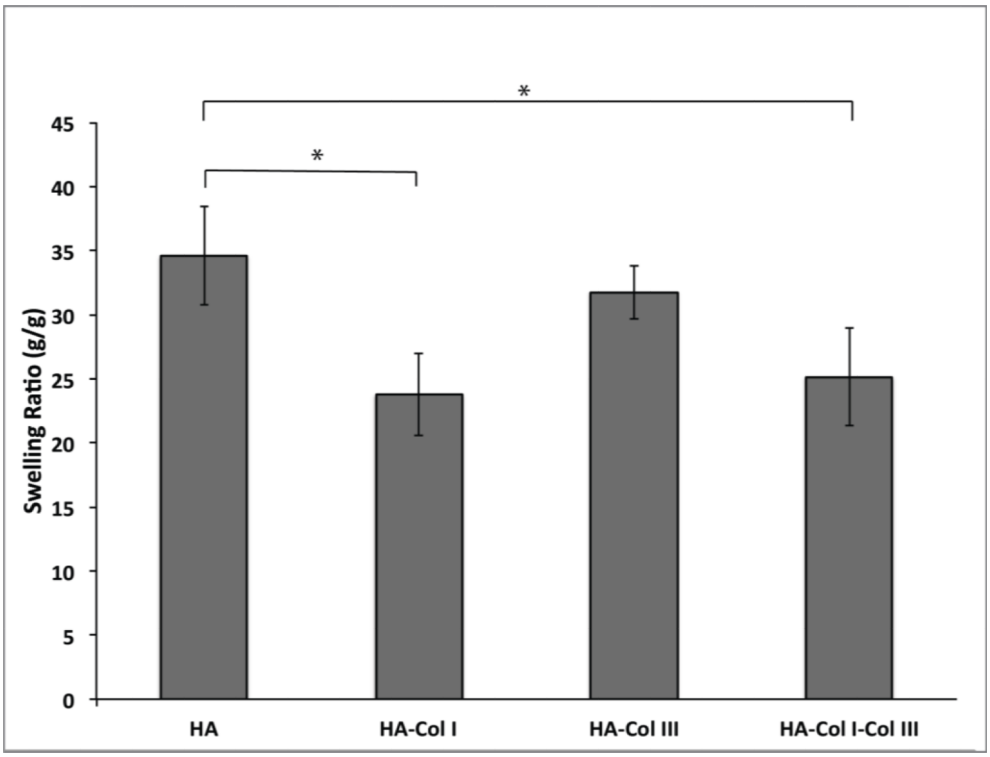

Figure 3–3. Swelling Ratios for Hydrogel Blends.

One way ANOVA indicated that the type of hydrogel affected swelling ratios for n=3 hydrogels. Tukey's post-hoc test revealed that the addition of type I collagen significantly decreased the swelling ratios of the HA hydrogels.

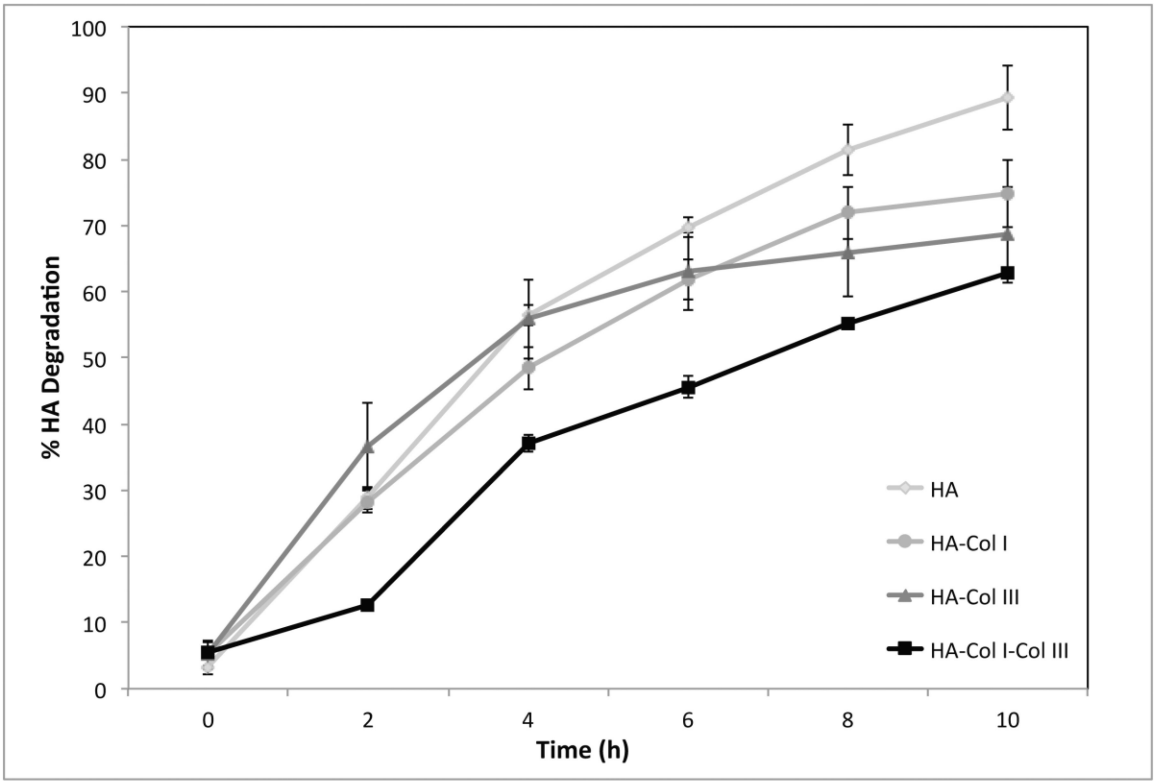

Figure 3–4. Hyaluronidase Degradation of Thiolated HA in Hydrogels Blends.
Reduced degradation of HA was seen with the addition of collagen, correlating with the reduced swelling ratios of the hydrogels containing HA and collagen. Error bars depict standard deviation for n=3.

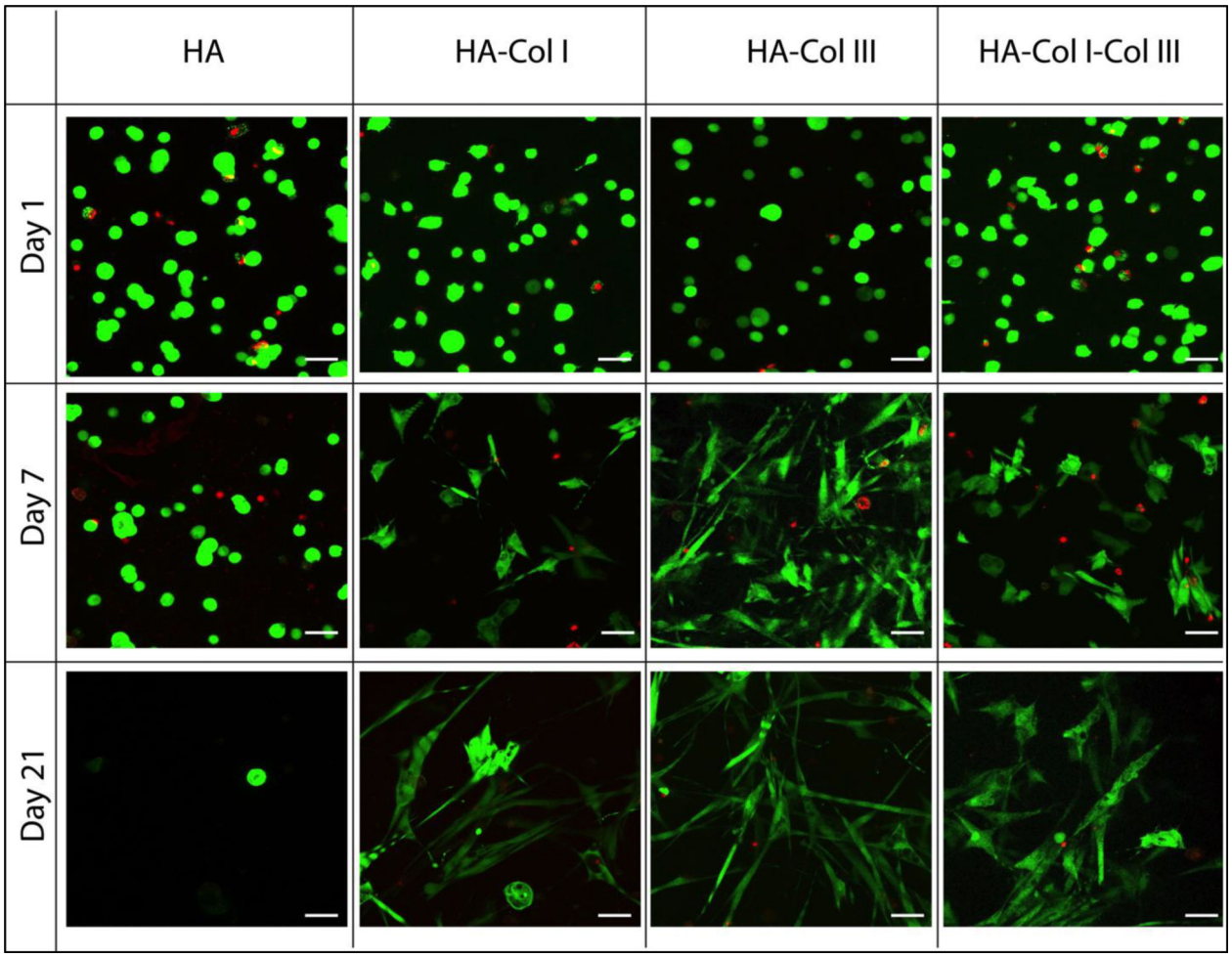

Figure 3–5. Live Dead Viability Assay on Fibroblasts Encapsulated in Hydrogel Blends Over 21 Days.

Calcein AM in green stains live cells and ethidium homodimer in red stains dead cells. Images are represented as maximum intensity projections of Z-stacks imaged within a total thickness of 570 μm. Majority of the cells in the HA-Col blend hydrogels remained viable even at the end of 21 days of culture. Scale bar = 50 μm.

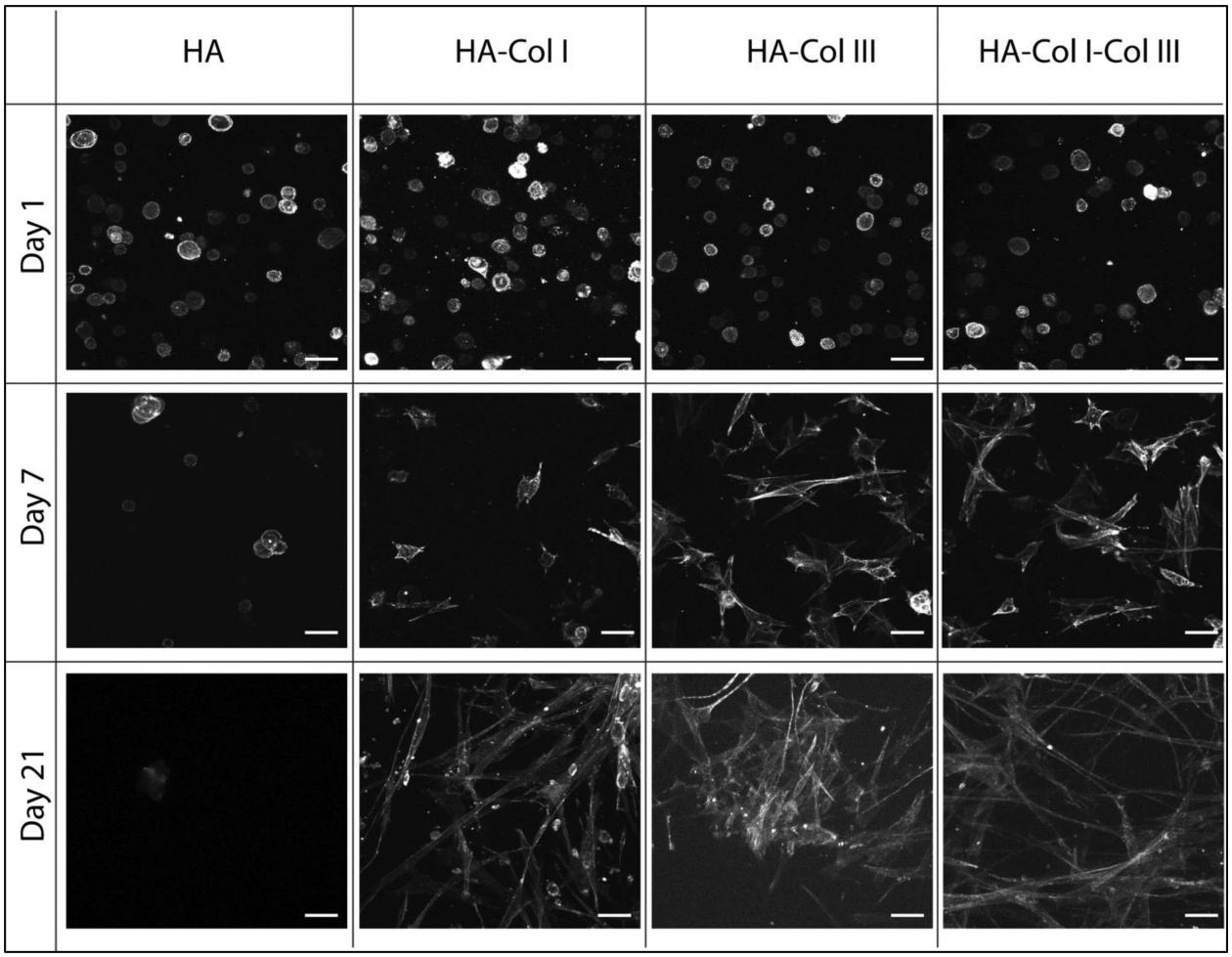

Figure 3–6. F-actin Staining in Hydrogel Blends.

Phalloidin was used to stain the actin cytoskeleton of fibroblasts encapsulated in the different hydrogel blends. Images are represented as maximum intensity projections of Z-stacks imaged within a total thickness of 570 μm. At 21 days, cells develop extensive cytoskeletal networks as seen by their spreading in the HA-Col blend hydrogels. Scale bar = 50 μm.

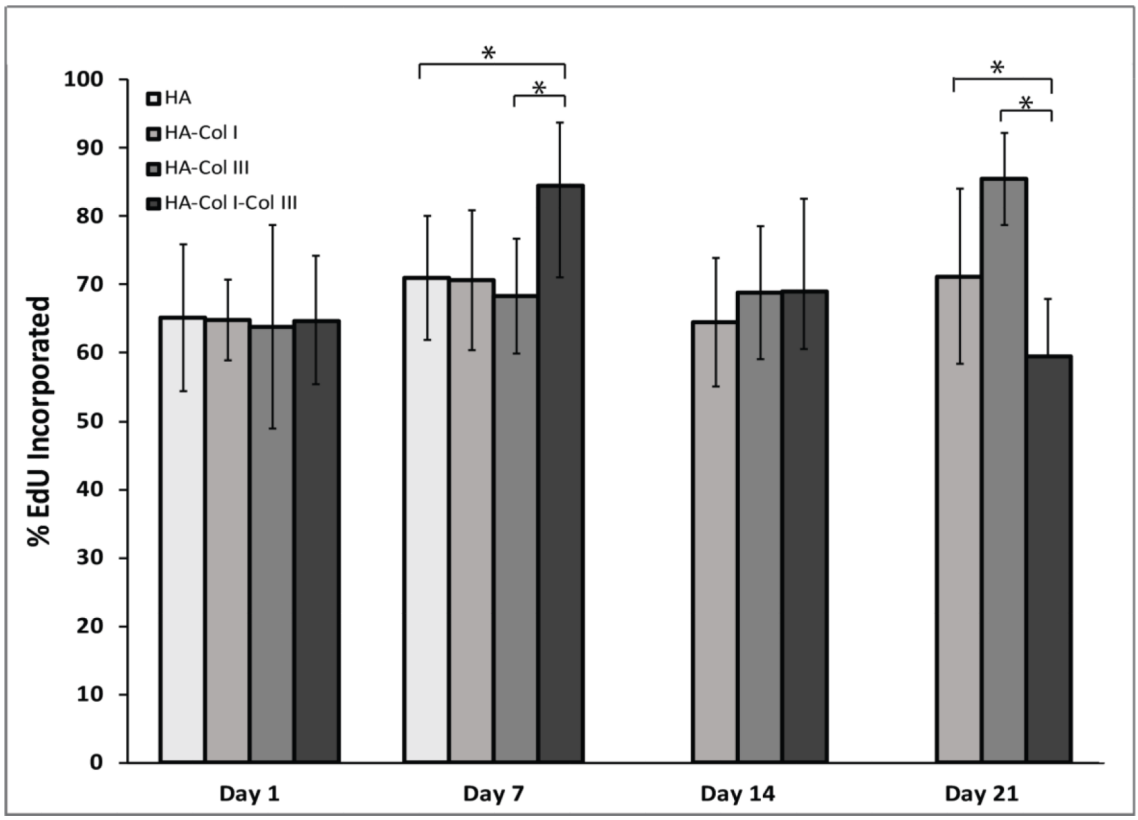

Figure 3–7. Percent Proliferation of Fibroblasts Encapsulated in Hydrogel Blends.

Fibroblasts remain in a proliferative state at the end of 21 days of culture in HA-Col blend hydrogels as seen by the incorporation of EdU into the newly synthesized DNA. Error bars depict standard deviation. One way ANOVA with Tukey's post-hoc test was used to determine statistical significance in percentage of EdU incorporation in encapsulated fibroblasts from n=9 images (*$P < 0.05$).

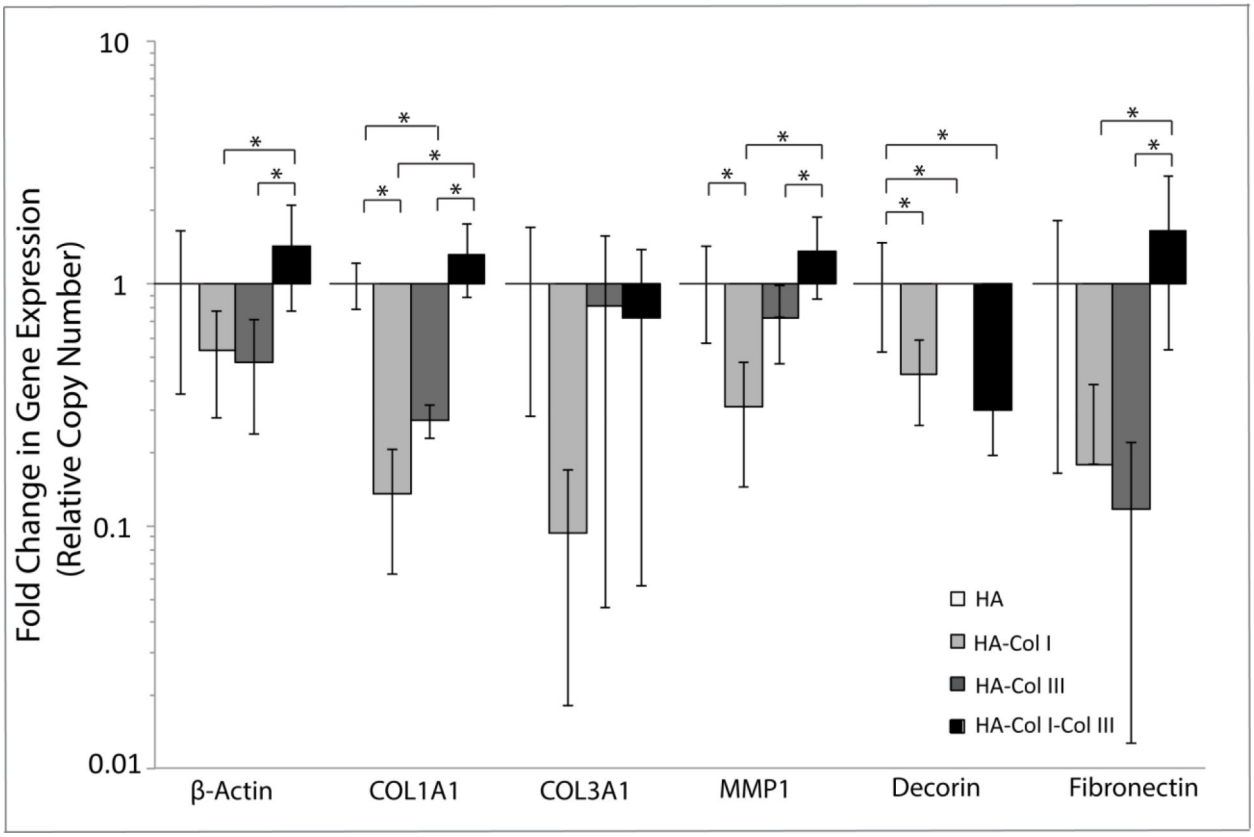

Figure 3–8. Gene Expression of Fibroblasts Encapsulated in Different Hydrogel Blends.

Fold changes in fibroblast ECM related genes when normalized to HA alone gels show that cells respond differently to their microenvironments. One way ANOVA with Tukey's post-hoc test was used to determine differences in gene expression between cells encapsulated in different hydrogel blends. Error bars represent uncertainty propagation of normalized data for n=3. (*P < 0.05).