

Mechanically Robust and Bioadhesive Collagen and Photocrosslinkable Hyaluronic Acid Semi-Interpenetrating Networks

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In this work, we present a class of hydrogels that leverage the favorable properties of the photo-cross-linkable hyaluronic acid (HA) and semi-interpenetrating collagen components. The mechanical properties of the semi-interpenetrating-network (semi-IPN) hydrogels far surpass those achievable with collagen gels or collagen gel-based semi-IPNs. Furthermore, the inclusion of the semi-interpenetrating collagen chains provides a synergistic mechanical improvement over unmodified HA hydrogels. Collagen–HA semi-IPNs supported fibroblast adhesion and proliferation and were shown to be suitable for cell encapsulation at high levels of cell viability. To demonstrate the utility of the semi-IPNs as a microscale tissue engineering material, cell-laden microstructures and microchannels were fabricated using soft lithographic techniques. Given their enhanced mechanical and biomimetic properties, we anticipate that these materials will be of value in tissue engineering and three-dimensional cell culture applications.

Introduction

FABRICATING ROBUST biocompatible three-dimensional (3D) matrices that support cell growth and tissue formation is a prerequisite for many cell culture and tissue engineering applications.^{1,2} Hydrogels are a potentially useful scaffold material for tissue engineering, as well as for 3D tissue culture because of their biocompatibility, high water content, and 3D nature.^{3–9} One disadvantage of many hydrogels is that they are mechanically weak. Because mimicking the mechanical aspects of natural tissues can be used to enhance the functionality of engineered tissues, the development of hydrogels that are more mechanically robust may be beneficial for various biological and biomedical applications.¹⁰

The *in vivo* extracellular matrix (ECM) is a hydrogel-like structure comprising different biopolymers with a wide range of biological, chemical, and mechanical properties. The organization and assembly of these components at the molecular level gives the ECM its widely ranging properties, which are unique for each tissue type.

Collagen and hyaluronic acid (HA) are two major components of the ECM that are commonly used for tissue engineering. Collagen is a natural ECM containing many cell-signaling domains¹¹ that can be gelled without chemical

modifications.¹² HA is a glycosaminoglycan heteropolysaccharide^{13,14} that has been cross-linked to form gels through multiple chemical modification means,^{15–18} including the methacrylation of HA macromers (methacrylated HA (MeHA)) to produce ultraviolet (UV) cross-linkable polymer solutions.^{19,20} HA has previously been incorporated into composite hydrogels with synthetic poly(ethylene glycol) (PEG) components for cellular studies.²¹ Un-cross-linked HA has also been incorporated into collagen gels to increase the collagen gel elastic modulus.²² However, potential disadvantages in these two systems are that they include non-biodegradable synthetic materials (PEG) or lack desired mechanical systems (for collagen-based gels).

By generating composite hydrogels, it may be possible to reproduce the properties of natural ECM. One approach to creating composite materials is the fabrication of an interpenetrating network (IPN) or semi-IPN of polymers. A semi-IPN consists of a polymer network containing molecularly entangled chains of a second polymer.²³ It has been demonstrated that synthetic and non-biological IPN hydrogels can achieve an increase in strength, failure stress, and stiffness while maintaining elasticity.^{24,25} However, such IPNs are often non-degradable and not applicable for cell culture. To develop biologically derived IPNs, collagen gels containing

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other ECM components have been generated^{26,27} with modest improvements in mechanical robustness.²²

Here we present a class of novel hydrogels containing photo-cross-linkable HA and semi-interpenetrating collagen components, which exhibit synergistic mechanical properties that far surpass those achievable with collagen gel-based semi-IPNs. We demonstrate that the stiffness of the semi-IPNs can be engineered based on methacrylation and concentration of the HA. Furthermore, these biologically derived materials can support cell adhesion and proliferation and may be microengineered to control the resulting scaffold architecture. In this work, collagen–MeHA hydrogels were fabricated by mixing the pre-polymer solutions and sequentially cross-linking each of the components. We focused on using HA-based gels because we hypothesized that it would provide better mechanical control than collagen.

Materials and Methods

MeHA synthesis and chemical analysis

MeHA was synthesized as previously described.¹⁹ Briefly, methacrylic anhydride was reacted with a 1 wt% (% of total solution mass) solution of HA (molecular weight (MW) = 75 kDa; Lifecore Hyaluronan Division, Chaska, MN) for 24 h to produce MeHA chains. The MeHA solution was dialyzed for 48 h and lyophilized for 72 h. The lyophilized product was then dissolved at a stock concentration of 10 wt% in phosphate buffered saline (PBS) and further diluted to prepare each sample. Degree of methacrylation (methacrylation percentage) was determined using ¹H-nuclear magnetic resonance (NMR) spectroscopy. Spectrums were generated using a 600-MHz NMR spectrometer, and degree of methacrylation was calculated as the ratio of methacrylate group protons (6.6–5.6 ppm integration) to HA polysaccharide backbone protons (4.2–3.0 ppm integration) normalized according to the number of protons per group.

Collagen–MeHA IPN fabrication

Freeze-dried collagen type I from rat tail (BD Biosciences, San Jose, CA) was dissolved in 0.02 M acetic acid (Fluka, Milwaukee, WI) at a concentration of 15.7 mg/mL. Collagen–MeHA IPNs were fabricated from the 15.7-mg/mL collagen and the 10 wt % MeHA prepolymer stock solutions. The collagen solution was neutralized and diluted with deionized water to a concentration such that, when mixed with a corresponding amount of MeHA, the final collagen solution was 0.4 wt%. After preparation of the collagen solution, MeHA prepolymer was pipetted into the collagen solution to produce the desired concentration of MeHA. MeHA solutions without collagen were also prepared by diluting the 10 wt% MeHA. Prepolymer solutions were stirred overnight at 4°C before addition of 1.5 wt% (relative to mass of total solution) of photoinitiator solution (33 wt% Irgacure 2959 (Ciba, Tarrytown, NY) dissolved in methanol) and stirred for 5 additional min. The resulting mixtures were then poured onto prefabricated poly(dimethylsiloxane) (PDMS) (Dow Corning Corp., Midland, MI) molds to achieve consistent sample sizes and then irradiated with UV light for 180 s and placed in a 95% air/5% carbon dioxide (CO₂), 100% humid 37°C incubator for 2 h before being removed from the PDMS molds for further studies. UV exposures were performed using an EXFO OmniCure Series 2000 (Mississauga, Canada) at 200 mW/cm².

Fluorescent collagen imaging

Collagen distribution within hydrogels was analyzed by quantifying the distribution of fluorescein isothiocyanate-labelled collagen (FITC-collagen, Stamford, CT) on a Nikon Eclipse TE2000-U microscope at exposure and emission wavelengths of 495 and 520 nm, respectively. For top cross-sectional views, the central 400-x400-pixel area was selected to minimize edge effects.

Mechanical testing

Hydrogel discs ($n = 5$, 2 mm height, 8 mm diameter) were fabricated in PDMS molds as described above. After initial incubation, the hydrogels were removed from the molds and allowed to equilibrate in PBS at 37°C for 24 h. Hydrogels were then compressed in the direction normal to the circular face of the disc on an Instron 5542 mechanical tester, (Norwood, MA) at a rate of 20% per min until mechanical failure occurred. The compressive modulus, defined as the slope of the linear region of the stress–strain curve of a material under compression, was calculated from the linear regime in the 10% to 15% strain range. Fracture points were taken as the stress peak after which a significant (>10%) decrease in stress occurred.

Cell culture, seeding, and encapsulation

NIH-3T3 cells were cultured in Dulbecco's modified Eagle medium supplemented with fetal bovine serum and penicillin–streptomycin (Gibco) in a 95% air/5% CO₂, 100% humid 37°C incubator. For surface adhesion studies, 8-mm-wide×1-mm-thick hydrogel discs were placed in PDMS wells and covered with 200 μL of cell suspension containing 250,000 NIH-3T3 cells/mL. Hydrogel surfaces were imaged at 6, 24, and 72 h. For encapsulated cell viability studies, NIH-3T3 cells were incorporated into prepolymer solutions at 2×10⁶ cells/mL. UV exposure was performed at 4 mW/cm² for 10 min. After 2 h incubation, cells were assayed using a calcein/homodimer live/dead assay (Invitrogen, Carlsbad, CA). All micromolding of cell-laden hydrogels was performed using soft lithography from PDMS masters. PDMS micromolds were fabricated from photoresist (SU-8 2100 Microchem, Newton, MA) patterned silicon wafers.

Statistical analysis

Statistical significance was evaluated using the Student *t*-test. *P*-values less than 0.05 were determined to be statistically significant.

Results and Discussion

Visualization of collagen distribution and network microstructure

To analyze the distribution of components in the composite gels, we visualized the distribution of fluorescent components in the gels. Because HA and collagen are highly hydrophilic molecules, we did not anticipate a significant amount of phase separation. FITC-collagen was mixed with MeHA using repeated pipetting (well-mixed) and compared with an unmixed sample (unmixed) post-UV treatment. Fluorescent intensity plots are shown for the well-mixed hydrogels (Fig. 1A, E) and the unmixed hydrogels

(Fig. 1B, F). Top-view and cross-sectional images indicated that the FITC-collagen and MeHA were uniformly distributed within the well-mixed hydrogels. To control for MeHA autofluorescence, MeHA without FITC-collagen was used (Fig. 1C, G), and FITC-collagen without MeHA was used as a positive control (Fig. 1D, H). As expected, FITC-collagen samples without MeHA were uniformly bright. By comparing the images of the FITC-collagen–MeHA hydrogels with the images of the MeHA-only hydrogels, it can be seen that the FITC-collagen remains in the hydrogels during UV cross-linking and collagen gelling. Furthermore, by com-

paring the fluorescent images of the well-mixed with the unmixed FITC-collagen–MeHA hydrogels, no detectable phase separation was observed. To further analyze the physical structure of the collagen–MeHA hydrogels, scanning electron microscopy (SEM) was performed. As shown, 0.4 wt% collagen gels generated fibrous structures (Fig. 1I, L), whereas MeHA-only hydrogels consisted of sheets or flakes (Fig. 1J, M) and were less smooth than the collagen fibers. In contrast, collagen–MeHA composite gels were highly porous and contained elements of both types of gels (Fig. 1K, N).

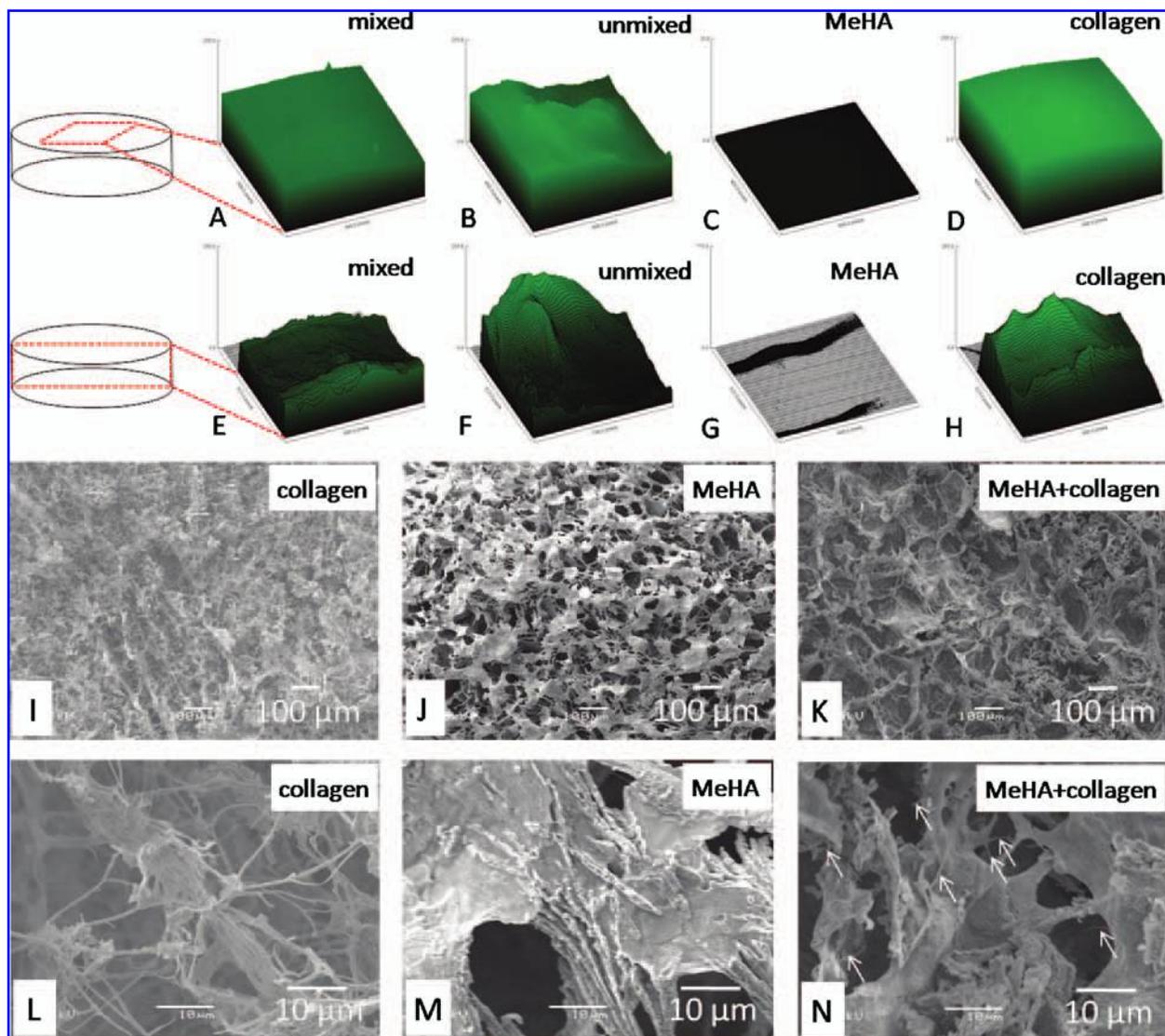


FIG. 1. Visualization of collagen and methacrylated hyaluronic acid (MeHA) mixtures. The homogeneity of well-mixed collagen–MeHA (A, E), unmixed collagen–MeHA (B, F), MeHA only (C, G), and collagen only (D, H) were examined by mixing a small fraction of fluorescein isothiocyanate–conjugated collagen into the collagen stock. Top views (A–D) demonstrate that the well-mixed collagen and MeHA resulted in a more-uniform collagen distribution than that of the unmixed collagen and MeHA, comparable with that of the collagen-only control. The same trend is observed in cross-sectional views (E–H) (views are 2.5 mm × 2.5 mm). As expected, the MeHA-only hydrogels (B, F) did not fluoresce. Also, the network microstructures were examined using scanning electron microscopy at 100× (I–K) and 2000× (L–N). Collagen-only networks (I, L) displayed a highly fibrous structure, MeHA-only networks (J, M) were made of flaky porous sheets, and elements of the fibrous collagen structure and the MeHA flakes were present in the well-mixed collagen–MeHA IPNs (K, N). Color images available online at www.liebertonline.com/ten.

Compression and fracture analysis of collagen-MeHA semi-IPNs

To characterize the effects of hydrogel composition and methacrylation rate on mechanical strength of the hydrogels, three sets of composite gels were subjected to compressive testing using an Instron 5542 mechanical tester. The three sets contained MeHA from separate syntheses with low (24%), medium (35%), and high (63%) degrees of methacrylation as determined using $^1\text{H-NMR}$. To vary the gel composition, MeHA concentrations of 2.5 wt%, 5.0 wt%, and 7.0 wt% were used to form composite gels with 0.4 wt% collagen. To assess the effects of IPN compositions, MeHA hydrogels (2.5, 5.0, and 7.0 wt%) served as a control. Collagen-only control gels were extremely weak and consequently fell below the sensitivity of the testing device, as also validated by previous studies.²⁸

As shown, collagen-MeHA hydrogels exhibited greater compressive moduli than their MeHA counterparts for nearly all tested conditions (Fig. 2A). Additionally, low-methacrylation 2.5 wt% MeHA with collagen had a consistent, measurable compressive modulus, whereas 2.5 wt% MeHA fell below the sensitivity threshold for testing. To summarize, not only were the collagen-MeHA composite gels more mechanically robust than collagen gels, but also, in nearly all cases, the addition of a small concentration of collagen provided a synergistic effect that produced a gel more robust than the sum of MeHA and collagen controls. Consistent with findings in other hydrogel materials,²⁹ greater methacrylation percentages resulted in an increase in compressive moduli of the gels. Unlike previous studies that demonstrated greater mechanical robustness in IPN or semi-IPN materials, which were based on non-biological materials,^{24,25} these experiments show that the mechanical improvement in biologically derived MeHA hydrogels can be achieved by simply incorporating a small quantity of a bioactive material: collagen. Furthermore, we hypothesized that the cell-adhesive nature of collagen will also influence biological activity of the gels more than the incorporation of a non-cell-adhesive material such as PEG.³⁰ Finally, our hydrogels can achieve a much greater level of stiffness through HA cross-linking than is possible with collagen hydrogels, even with incorporated un-cross-linked polymers.²²

To analyze the mechanical behavior of the materials under strain, fracture stress points were analyzed for the composite and control hydrogels (Fig. 2B). Fracture points were taken as the stress peak after which a significant (>10%) decrease in stress occurred. Generally, the hydrogels entered the plastic deformation region just before fracturing. As predicted, greater methacrylation percentages and MeHA concentrations resulted in greater fracture stress. For high methacrylation percentage MeHA, the 5.0 and 7.0 wt% collagen-MeHA hydrogels exhibited significantly higher fracture stresses than the MeHA gels. However, in most conditions, the fracture stress of composite hydrogels was similar to MeHA-only gels. This suggests that the presence of collagen may be less important than MeHA for determining gel fracture point. Taken together, the greater compressive modulus strength and higher fracture stress as a function of methacrylation indicates that this process may be useful for strengthening mechanically weak biological hydrogels for tissue engineering applications where mechanical strength is of significant importance.

To further understand the structure of the collagen-MeHA hydrogels, experiments were performed to isolate the effects of HA photo-cross-linking and collagen temperature-induced network formation. To examine the effect of collagen network formation on composite gel properties, the UV cross-linking step was performed, and the 37°C incubation step was omitted. Alternatively, to examine the effect of MeHA network formation, polymer solutions of collagen-MeHA and MeHA were prepared without UV exposure but with 37°C incubation. Inhibition of collagen fibrillogenesis had no significant effect on compressive modulus from gels that underwent 37°C incubation (Fig. 2C). As expected, MeHA solutions remained liquid. Collagen-MeHA solutions without UV formed mostly liquid gels with segments of thin collagen networks dispersed throughout the mixture with weak mechanical properties (Fig. 2D). The similarity between incubated and non-incubated hydrogels suggests that the MeHA cross-linking is a more important factor for mechanical stiffness than collagen network formation. If the collagen formed a highly interconnected network within the MeHA network, the collagen network formation would be expected to have a significant mechanical effect. The lack of this feature suggests that the collagen-MeHA hydrogels are semi-IPNs. The observation that non-UV-exposed collagen-MeHA had a weak structure further supports the conclusions that MeHA is the dominant structural component of the hydrogels and that the materials are semi-interpenetrating.

NIH-3T3 cell adhesion and proliferation on semi-IPN surfaces

To determine the cell adhesiveness of collagen-MeHA semi-IPNs and their utility as a tissue scaffold material, NIH-3T3 cells were seeded at high density on the surface of the semi-IPNs, MeHA, and collagen gels (medium MeHA methacrylation was analyzed). The surfaces were imaged at 6, 24, and 72 h after seeding and quantified to determine 2D cell density (Fig. 3A). After 6 h, significant differences between cell adhesiveness of different gels were observed. Specifically, it was seen that MeHA hydrogels did not result in significant adhesion of cells at lower MeHA concentrations. Higher MeHA concentrations resulted in greater cell adhesion, potentially due to greater protein adsorption caused by lower water content and greater hydrophobicity, as previously published.^{31,32} At 24 and 72 h, lower-concentration MeHA showed lower cell density, with most cells detaching from the surfaces and forming aggregates. At 72 h, essentially all cells on 2.5 wt% MeHA had detached into cell aggregates (Fig. 3B). The 5.0 wt% MeHA surface consisted of mostly detached cell aggregates at 72 h, although a few patches of adhered cells remained (Fig. 3C). In contrast, 2.5 and 5.0 wt% collagen-MeHA hydrogels exhibited excellent cell adhesion at 24 and 72 h (Fig. 3E, F). At 24 h, cell adhesion and proliferation for 5.0 and 7.0 wt% MeHA semi-IPNs was comparable to the collagen gels. Although the cell density for collagen gels at 72 h was higher than 5.0 and 7.0 wt% semi-IPNs, the semi-IPNs were entirely confluent with cells, and it is likely that the higher cell density in collagen gels was due to collagen gel contraction, which was visibly noticeable, as previously documented.^{26,33} The combination of the cell adhesive properties of collagen and a mechanical stiffness sev-

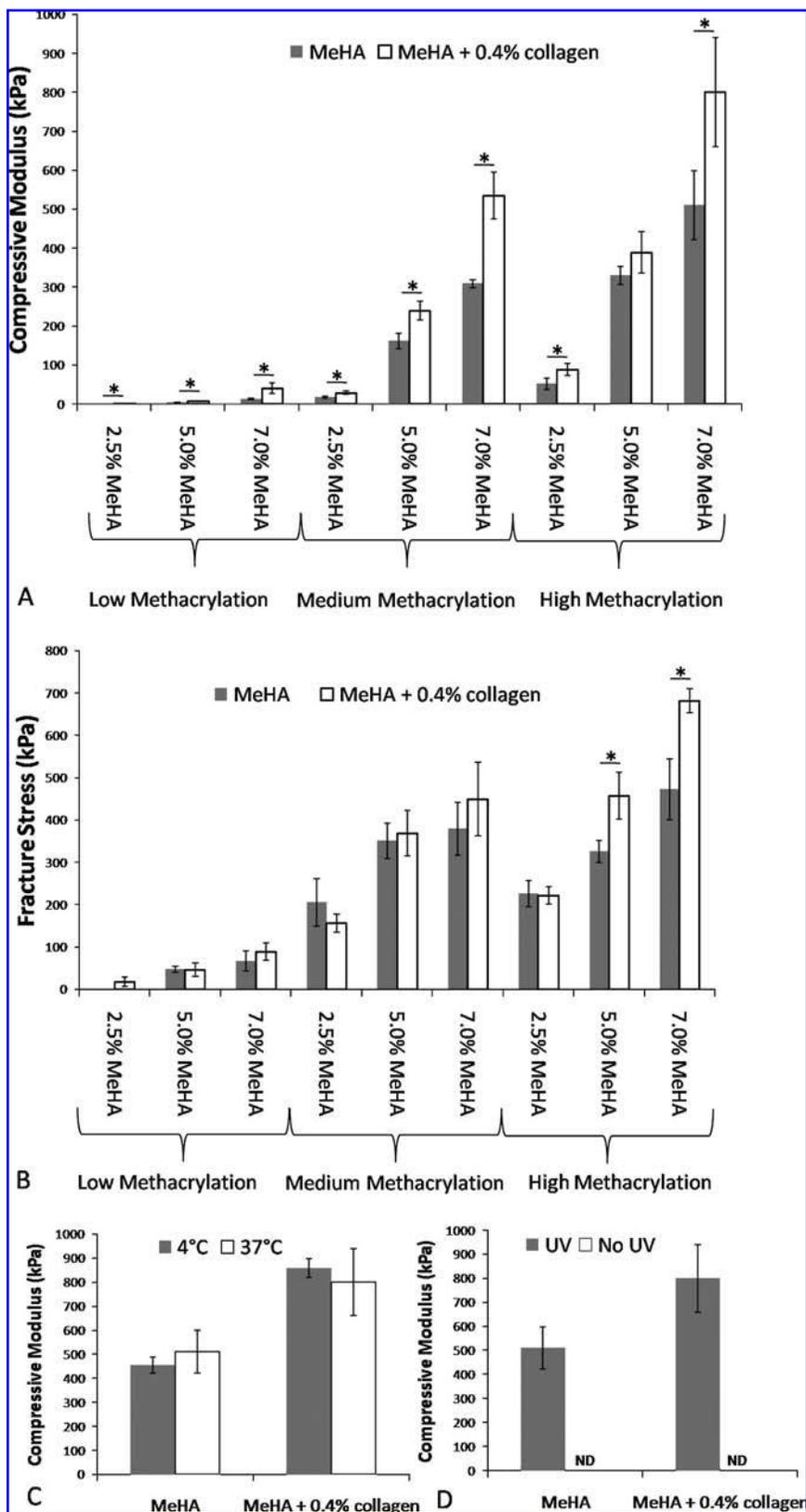
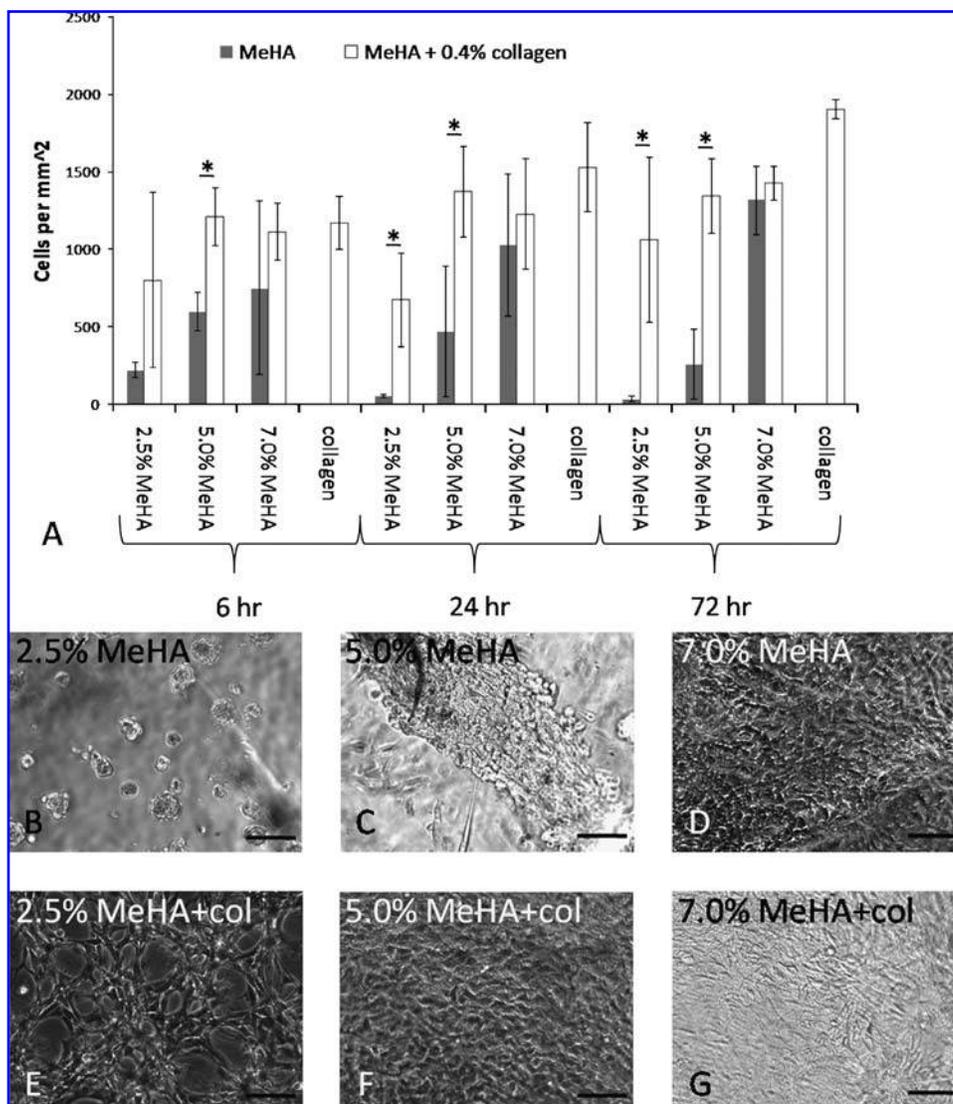


FIG. 2. Mechanical properties of collagen-methacrylated hyaluronic acid (MeHA) interpenetrating networks (IPNs) using MeHA with low, medium, and high methacrylation. The addition of 0.4 wt% collagen to each concentration of MeHA resulted in a significant increase ($*p < 0.05$) in compressive modulus (10–15%) in most cases (A). The collagen-only gels were below the sensitivity of the testing device and are not shown. Fracture point stress was also measured (B) and showed significant differences only when the highly methacrylated MeHA was used. Inhibition of collagen cross-linking in collagen-MeHA by maintaining gels at 4°C showed no difference from gels incubated at 37°C (C). Collagen-MeHA prepolymer solutions placed directly in 37°C incubation without ultraviolet exposure (D) resulted in a liquid-like gel containing segments of collagen networks interspersed that were not detectable (ND) during mechanical testing.

FIG. 3. NIH-3T3 cell adhesion on collagen-methacrylated hyaluronic acid (MeHA) semi-interpenetrating network (IPN) hydrogel surfaces. NIH-3T3 cells were seeded on collagen-MeHA, MeHA-only, and collagen-only gels. Gel surfaces were imaged at 6, 24, and 72 h after seeding, and surface cell densities were determined (A). Representative images of gel surfaces at 72 h are shown (B–G). Cell adhesion on unmodified gels was seen only at high concentrations. *Statistically significant differences ($p < 0.05$). Scale bar equals 100 μm .



eral orders of magnitude higher than collagen gels suggests the utility of collagen-MeHA semi-IPNs as a tissue engineering material. Although block-co-polymer HA-gelatin hydrogels³⁴ have been previously fabricated, to our knowledge, the formation of collagen-MeHA semi-IPNs has not been previously reported.

Cell encapsulation and microengineering of semi-IPNs

To further characterize the potential of the material for use in tissue engineering applications, the viability of encapsulated cells and the micromolding of cell-laden collagen-MeHA semi-IPN hydrogels were studied. NIH-3T3 fibroblasts were incorporated into the prepolymer solutions. After cross-linking, the cell-laden hydrogels were subjected to a calcein-homodimer cell-viability assay (Fig. 4A). Cells encapsulated in 2.5, 5.0 and 7.0 wt% MeHA with collagen showed high levels of cell viability (>75–85%) and were not statistically less than MeHA and collagen gels (with photoinitiator and UV) controls. Although there was less viability than in collagen without photoinitiator and UV, it is likely

that this could be eliminated for lower wt% solutions by reducing photoinitiator concentration.³⁵ To assess the processability of the semi-IPN hydrogels for microscale tissue engineering, mouse embryonic fibroblasts were incorporated into IPN prepolymer solutions (5.0 wt% MeHA and 0.4 wt% collagen) and molded with micropatterned poly (dimethylsiloxane) stamps. Microwells (Fig. 4B, C), microchannels, and other microstructures were formed with 300- μm average feature widths and depths. Furthermore, by using a two-step photo-cross-linking process, we were able to form and perfuse microchannels within the collagen-MeHA hydrogels (Fig. 4D). These microchannels are potentially advantageous for future microfluidic research and tissue engineering applications. Microfluidic devices could be made out of the collagen-MeHA hydrogel material, which would facilitate nutrient and cell-growth-factor exchange in 3D tissue constructs that could be used for biological analyses and assays. In particular, because the diffusion limit in porous scaffolds is a major limitation on 3D tissue engineering construct size, developing microchannels is a potential strategy to overcome this challenge. Additionally, the

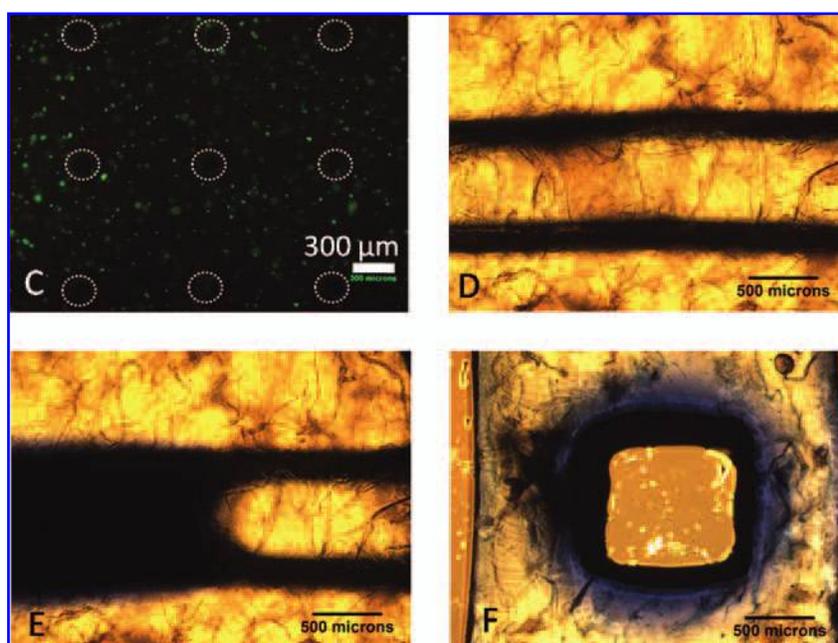
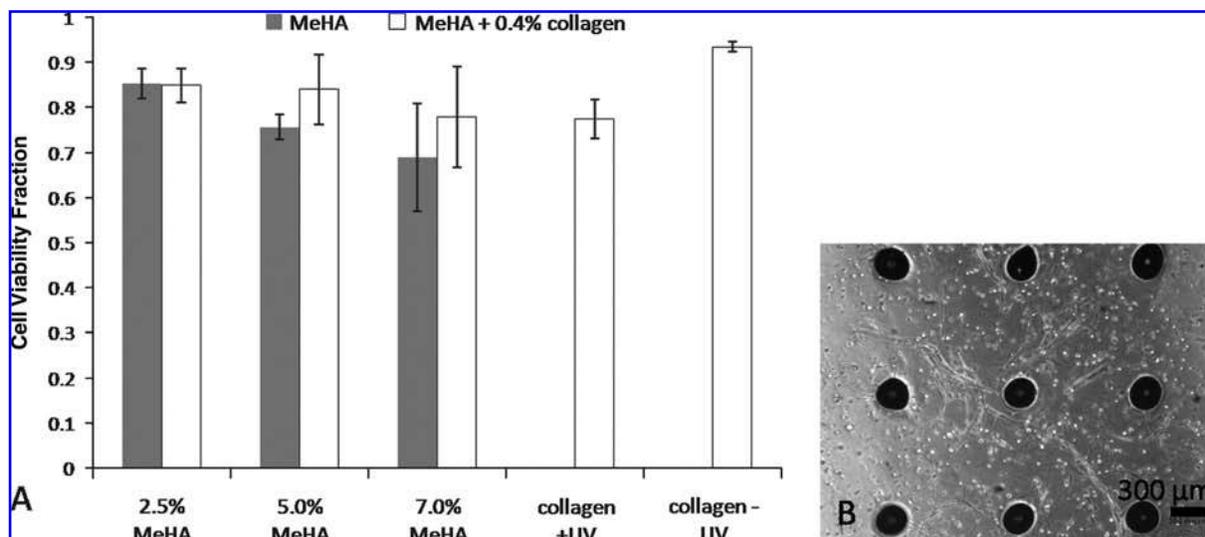


FIG. 4. Encapsulated cell viability and micromolding of collagen-methacrylated hyaluronic acid (MeHA) semi-interpenetrating networks (IPNs). After ultraviolet (UV) exposure and 2-h collagen gelling time, cell-laden polymer networks were soaked in calcein/homodimer live/dead stain to determine the viability of NIH-3T3 cells after encapsulation (A). To demonstrate the micromolding of cell-laden collagen and MeHA IPN hydrogels, mouse embryonic fibroblasts were mixed with 5.0 wt% MeHA with 4.1 mg/mL of collagen prepolymer and molded using a poly(dimethylsiloxane) stamp. Microwells with a diameter of 250 μm were fabricated with encapsulated mouse embryonic fibroblasts and analyzed for cell viability (B). The mouse embryonic fibroblasts exhibited high viability in the IPNs ($88.1 \pm 5.4\%$) (C). Collagen-MeHA microchannels were molded and sealed using a two-part UV cross-linking method (D). The gels were incubated, and the channel was subsequently perfused with trypan blue dye (E). After perfusion, a cross-section was taken, and the diffusion of the dye into the hydrogel was observed (F). Color images available online at www.liebertonline.com/ten.

tendency for the semi-IPNs to resist contraction under cellular tension suggests that the semi-IPNs would be advantageous for maintaining 3D structure.

Conclusion

We developed collagen-MeHA semi-IPNs with a number of potentially advantageous hydrogel properties, including

significantly better mechanical strength and biocompatibility. It was shown that the inclusion of collagen into the network created semi-IPNs with synergistically greater hydrogel compressive moduli across a variety of polymer concentrations and methacrylation percentages. Furthermore, the semi-IPNs were cell adhesive, exhibited high encapsulated cell viability, and could be molded to generate microscale structures. It is anticipated that, given their

biomimetic properties, these materials will be of value in tissue engineering and 3D cell-culture applications.

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Disclosure Statement

No competing financial interests exist.

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