Engineering 3D spatiotemporally dynamic hyaluronic acid hydrogels with heterogeneous mechanics to mimic idiopathic pulmonary fibrosis

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

Advisor: Dr. Steven R. Caliari, Departments of Chemical and Biomedical Engineering

Engineering 3D spatiotemporally dynamic hyaluronic acid hydrogels with heterogeneous mechanics to mimic idiopathic pulmonary fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive scarring disease that inhibits alveolar gas exchange through excess extracellular matrix (ECM) deposition in the interstitial space between alveoli and the surrounding capillary network. A lack of accurate preclinical models for IPF has contributed to the absence of a curative treatment for the disease which often becomes fatal within 3 years after diagnosis due to respiratory failure. Toward the objective of developing a preclinical model of IPF to gain insight into the cellular mechanisms responsible for IPF and for the testing of therapeutics, we developed a 3D hyaluronic acid (HA) hydrogel model with precise spatiotemporal control over matrix mechanics such as elasticity and viscoelasticity. Through light-mediated crosslinking, we were able to photopattern hydrogels to selectively introduce areas of secondary crosslinking and stiffen the hydrogel in a heterogeneous manner. This heterogeneity mimics the formation of fibrotic nodules seen in native tissue during fibrogenesis in which islands of stiffness surrounded by healthy, relatively unaffected tissue form. Pattern fidelity was confirmed through the thickness of 3D hydrogels by visualization with a fluorescently labeled peptide and through nanoindentation tests of hydrogel mechanics. Degradability was also engineered into the hydrogel using a matrix metalloproteinase (MMP)-degradable peptide so that cells encapsulated in 3D hydrogels could remodel their surrounding network. Human lung fibroblasts (HLFs), the main mediators of IPF, were encapsulated in hydrogels with heterogeneous mechanics to observe initial cell behaviors. Encapsulations showed that the photopatterning process did not negatively impact cell viability. Overall, the ability to successfully fabricate 3D hydrogels with a high degree of control over mechanics such as elasticity and viscoelasticity represents an important step in the development of more accurate preclinical models.

Keywords: Idiopathic pulmonary fibrosis, heterogeneity, viscoelasticity

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease characterized by tissue scarring that can become fatal due to respiratory failure. Patients diagnosed with IPF have a median survival of less than 3 years after diagnosis with 40 percent of fatalities resulting from respiratory failure. Although IPF is the most common and aggressive form of interstitial lung disease, current therapies have proven ineffective due to a lack of knowledge surrounding

how cells sense and respond to dynamic environmental cues.[1]

Cells and their surrounding tissue environment participate in dynamic bidirectional signaling that regulates both the properties of the extracellular matrix (ECM) and cell behavior. Mechanisms such as mechanotransduction, the conversion of mechanical stimuli to biochemical signals, allow cells to sense cues presented by the ECM and respond accordingly. This signaling becomes especially critical during diseased states such as IPF in which a heterogeneous pathological scarring process results in excess collagen deposition by cells. During IPF progression, healthy lung tissue mechanics will become stiffer and lose viscoelasticity. This progression takes place in a non-uniform manner with the formation of fibroblastic foci surrounded by areas of soft, relatively unaffected tissue.[2,3]

Accurate *in vitro* disease modeling is important in informing therapeutic design through its ability to explore a broad range of variables that contribute to disease progression as well as the capability to perform high-throughput drug screening that would be unsuitable with *in vivo* animal models. Hydrogels are water swollen polymer networks that are often used to model normal and diseased tissues due to their ability to mimic soft tissue properties.[4] However, many current models are two-dimensional (2D), non-physiologically stiff, and have homogenous mechanical properties – all traits which fail to portray the native 3D tissue microenvironment. Additionally, the majority of models fail to incorporate time-dependent properties such as viscoelasticity, despite its key role in mediating in cell behavior. Previous work has shown that incorporating viscoelasticity into hydrogels impacts cell responses such as spreading, focal adhesion organization, proliferation, and differentiation when compared to solely elastic models. [5-10]

Here, we designed a 3D phototunable viscoelastic hydrogel system that allows for independent tuning of mechanical properties implicated in healthy and diseased lung tissue such as stiffness and viscoelasticity properties. Using this approach allowed for high spatiotemporal control over matrix properties to introduce heterogeneous mechanics to mimic the formation of fibroblastic foci that occur during fibrogenesis.

Results

3D hydrogels were successfully fabricated while maintaining high spatiotemporal control over matrix mechanics

A three-dimensional (3D) phototunable viscoelastic hydrogel system capable of independently tuning stiffness and viscoelasticity, mechanical properties that are both implicated in healthy and diseased lung tissue, was designed. Hyaluronic acid (HA), a non-sulfated glycosaminoglycan found in native ECM, was used as the polymer backbone because of its inherent biocompatibility, chemical tunability, and involvement in disease processes such as wound healing. HA was functionalized with norbornene groups (NorHA) which have a high reactivity to thiyl radicals to allow for rapid and highly controllable thiol-ene addition with thiolated crosslinkers and peptides. Soft (2 wt% NorHA) and stiff (6 wt% NorHA) 3D hydrogels were fabricated by adding hydrogel precursor solutions to cut syringes. Hydrogel precursor solution then underwent ultraviolet (UV)-light mediated thiol-ene addition for 5 minutes in the presence of lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) photoinitiator (**Figure 1**). The biorthogonality of the system allows for the facile synthesis of various hydrogel formulations with high specificity by changing a single parameter such as crosslinker concentration or light intensity.

Viscoelasticity was introduced into the system via incorporation of reversible guest-host interactions between β-cyclodextrin-modified hyaluronic acid (CD-HA) and thiolated adamantane peptides (**Figure 1**). The physical guest-host interactions impart time-dependent viscous properties that allow stress relaxation to occur in response to mechanical strain. Cyclodextrin (CD) has a hydrophobic cavity with a high affinity for guest molecules such as adamantane, allowing non-covalent interactions in combination with covalent interactions between NorHA and dithiol crosslinkers. Soft (3 wt% NorHA-CD-HA) and stiff (7 wt% NorHA-CD-HA) viscoelastic NorHA-CD-HA hydrogels were fabricated by mixing CD-HA and thiolated adamantane peptides (Ad) to introduce the Ad-CD guest-host interactions, then mixing in NorHA, RGD, and degradable dithiol crosslinker. The precursor solutions were

photopolymerized in the same method as the elastic hydrogel groups.

Using a combination of phototunable covalent and supramolecular interactions, both stiffness and viscoelasticity were independently tuned through crosslinker density and light intensity. Degradability was introduced to the system via crosslinking matrix metalloproteinase (MMP)-degradable peptides to allow matrix remodeling by cells.

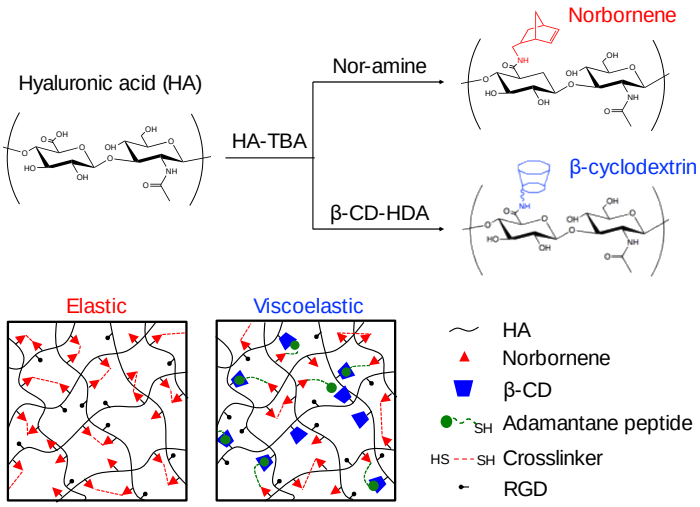

Figure 1. Schematic of hydrogel synthesis and network crosslinking. HA modified with functional groups permit the fabrication of hydrogels with covalent and physical, guest-host interactions. Elastic and viscoelastic hydrogels are all fabricated via photocrosslinking.

Degradability was engineered into 3D hydrogels using an MMP-degradable dithiol crosslinker

Human lung fibroblasts (HLFs), the main mediators of IPF, were encapsulated in hydrogels uniform mechanics to observe initial changes in cell morphology and to ensure the 3D hydrogel system supported a high cell viability. Encapsulations took place in hydrogels of varying stiffness and viscoelasticity over a 14-day culture period and the effect of mechanical cues on cellular behavior was evaluated. When observing encapsulated HLF behavior, a relatively rounded phenotype was maintained regardless of hydrogel mechanics.

To ensure that the consistently rounded morphology was not due to a lack of ability for HLFs to degrade the peptide sequence with which hydrogels were crosslinked, experiments were conducted to compare cell shape in hydrogels crosslinked with peptide sequences able to be degraded by different MMPs secreted by HLFs. Cells were encapsulated in elastic and viscoelastic hydrogels with soft and stiff mechanics. Two degradable sequences were tested (underlined region indicates the MMP-sensitive region): GCNSVPMSMRGGSNCG (cleavage between S and M residues) and GCNSPQGIWGSNCG (cleavage between G and I residues). Both HLFs and human mesenchymal stem cells (hMSCs) were encapsulated with hMSCs serving as a control as the activity of hMSCs using these peptide sequences has been well characterized. Cells were encapsulated and cultured in hydrogels for a 14-day culture period and viability, cell volume, and CSI were quantified by staining cells with a Live/Dead viability assay (calcein AM and ethidium homodimer to identify live and dead cells, respectively) then imaging with confocal microscopy (**Figure 2**).

Increased spreading by hMSCs over the 14-day culture period indicated that the MMP-degradable peptide was able to be degraded by cell-secreted MMPs. A high viability was maintained regardless of degradable peptide throughout the entire culture period. Cell volume and cell shape index (CSI), a measure of cell circularity in which a value of 0 indicates a line and a value of 1 indicates a perfect sphere, were also observed throughout the culture period to observe cell spreading behavior and compare between the degradable peptide sequences. More cell spreading was observed in the HLFs that were encapsulated in hydrogels crosslinked with the VPM degradable peptide sequence suggesting that this sequence was more responsive to MMPs secreted by HLFs (**Figure 2**). As such, all future experiments were conducted using the VPM degradable sequence as the MMP-degradable crosslinker.

Photopatterning of 3D hydrogels allows for the introduction of heterogeneous mechanics to mimic fibrotic nodule formation

Taking advantage of the high spatiotemporal control offered by the hydrogel system, heterogeneous mechanics were introduced to 3D hydrogels through photopatterning. Using photomasks to irradiate portions of a hydrogel with an initially low crosslinking density, secondary crosslinks could be selectively introduced with a degree of high spatial control (**Figure 3**). Initially soft elastic and viscoelastic hydrogels were fabricated using the same process outlined above. They were then incubated in a solution containing BSA to block non-specific binding, LAP photoinitiator, MMP-degradable dithiol cross linker, and a thiolated

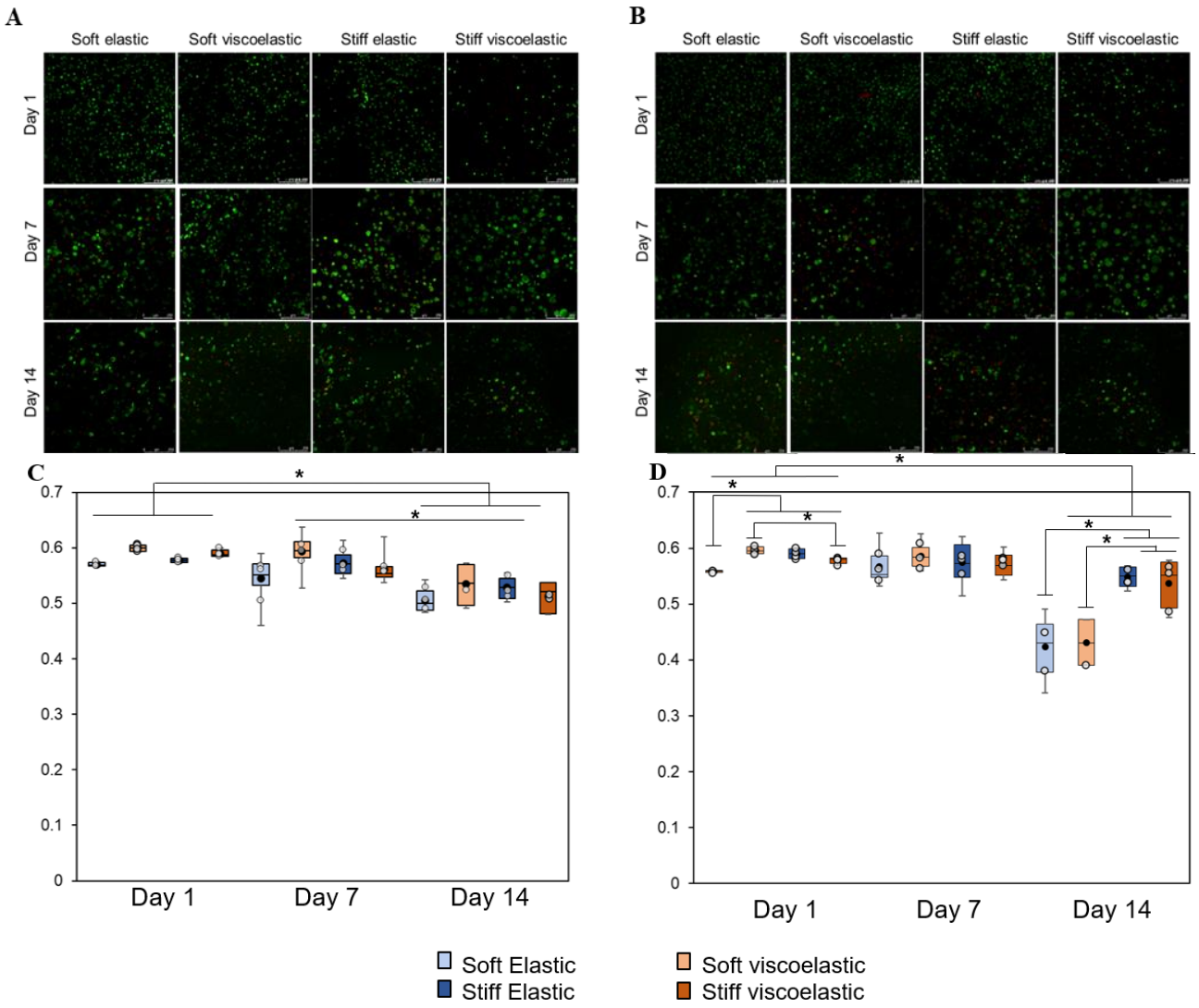

Figure 2. Preliminary 14-day HLF encapsulation in hydrogels with uniform mechanics to optimize hydrogel system. A) Representative images of HLFs encapsulated in hydrogels crosslinked with PQG degradable peptide sequence. Scale bars are 250 µm and images are max projections of a 400 µm z-stack with 10 µm spacing. B) Representative images of HLFs encapsulated in hydrogels crosslinked with VPM degradable peptide sequence. Scale bars are 250 µm and images are max projections of a 400 µm z-stack with 10 µm spacing C) CSI for HLFs encapsulated in hydrogels crosslinked with PQG degradable peptide where a value of 0 represents a line and a value of 1 represents a perfect sphere. Over the 14-day culture period, HLFs did not show differences in spreading between the mechanical groups. D) CSI for HLFs encapsulated in hydrogels crosslinked with VPM degradable peptide. HLFs showed more spreading in hydrogels crosslinked with this sequence, especially in groups with softer mechanics. *P < 0.05

fluorescent peptide for pattern visualization. A photomask with alternating regions of transparency and opacity was then placed over the initially soft hydrogel followed by UV irradiation to selectively stiffen those regions of the hydrogel that received a secondary irradiation.

Confocal microscopy visually confirmed pattern fidelity

A concern of scaling the photopatterning system from 2D to 3D was that the process would not effectively introduce heterogeneous mechanics through the full depth of 3D hydrogels. Conjugation of a fluorescent peptide to the patterned regions of the hydrogel during the secondary irradiation allows for confirmation of pattern fidelity via confocal microscopy. 400 µm z-stacks were imaged through hydrogels with images taken every 10 µm. Volume projections were performed to confirm pattern fidelity through the thickness of the hydrogel. Imaging demonstrated that the patterning process was effective

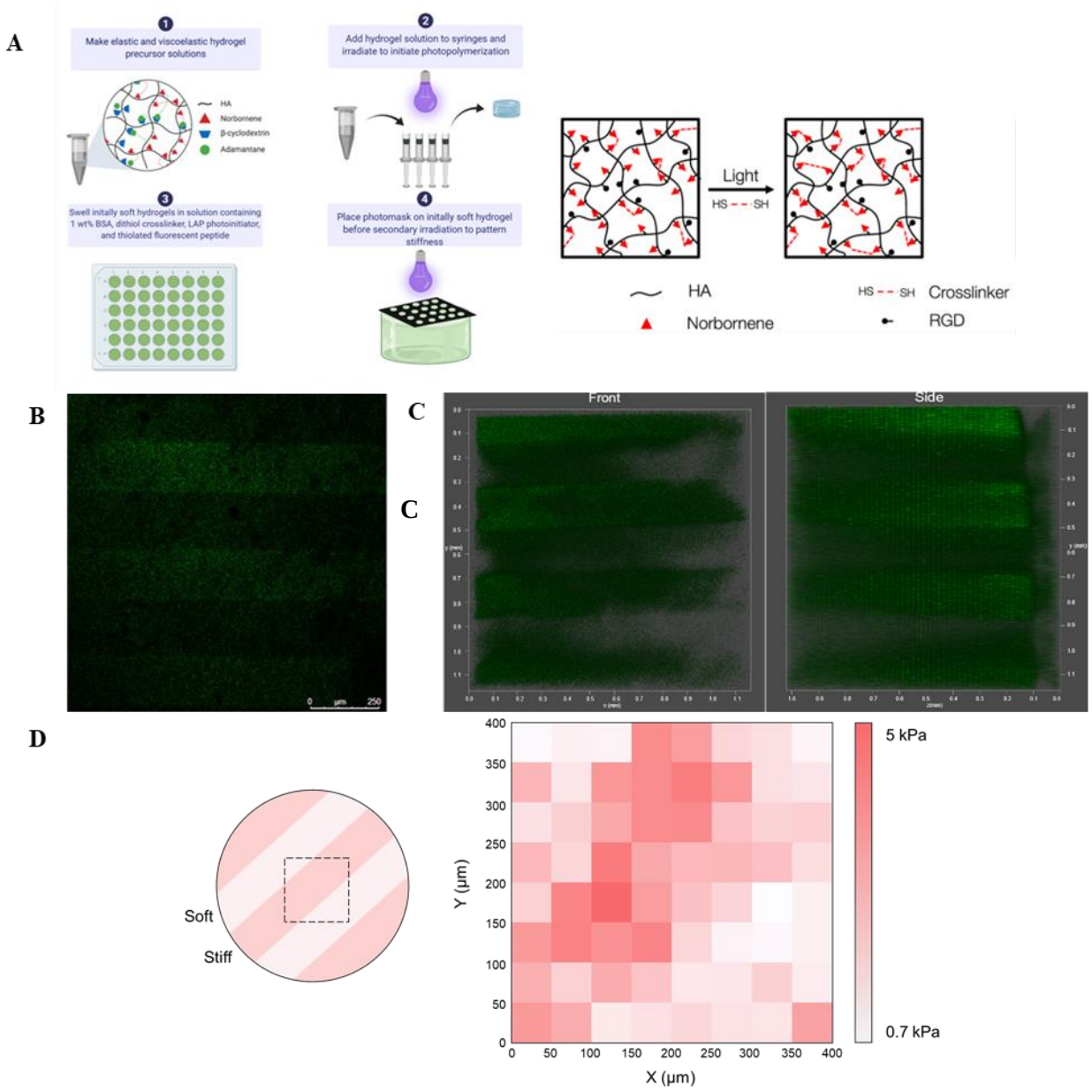

Figure 3. Photopatterning of 3D hydrogels to introduce heterogeneous mechanics. **A)** Photopatterning process used to selectively introduce areas of stiffness to hydrogels. A hydrogel with an initially low crosslinking density is incubated in a solution containing several components including additional dithiol crosslinker and a thiolated fluorescent peptide for pattern visualization. A photomask with alternating transparent and opaque regions is then placed on the hydrogel prior to a secondary UV irradiation. The secondary irradiation results in the introduction of covalent crosslinks via a light-mediated thiol-ene addition reaction in those regions of the hydrogel under a transparent portion of the photomask. **B)** Confocal microscopy for pattern visualization. Fluorescent regions of the images show the stiffer portions of the hydrogel. Image is one slice from a 400 μm z-stack with images taken every 10 μm. **C)** Volume projection of confocal microscopy images to confirm that patterning was able to penetrate through the depth of the hydrogels. Imaging confirmed pattern fidelity through 300 μm. **D)** Nanoindentation performed to mechanically characterize hydrogel heterogeneity. Young's moduli collected from a matrix scan shows pattern fidelity with softer and stiffer regions appearing to be about 200 μm apart.

through a thickness of about 300 μm of hydrogels (**Figure 3**).

Mechanical characterization of hydrogels via nanoindentation

To more robustly characterize heterogeneous mechanics photopatterned into hydrogels nanonindentation was performed. Hydrogels were fabricated using the same process as above then horizontally sectioned. An Optics11 Piuma nanoindenter with a 53.5 μm radius spherical borosilicate glass probe attached to a cantilever with a spring constant of 4.42 N/m was used for all tests.

An 8 by 8 matrix scan with indentations collected every 50 μm was performed to measure the Young's modulus across 400 μm of the hydrogel in the x-y direction. Matrix scans showed that the Young's modulus of the hydrogel ranged from 0.7 kPa to 5 kPa depending on the region of the

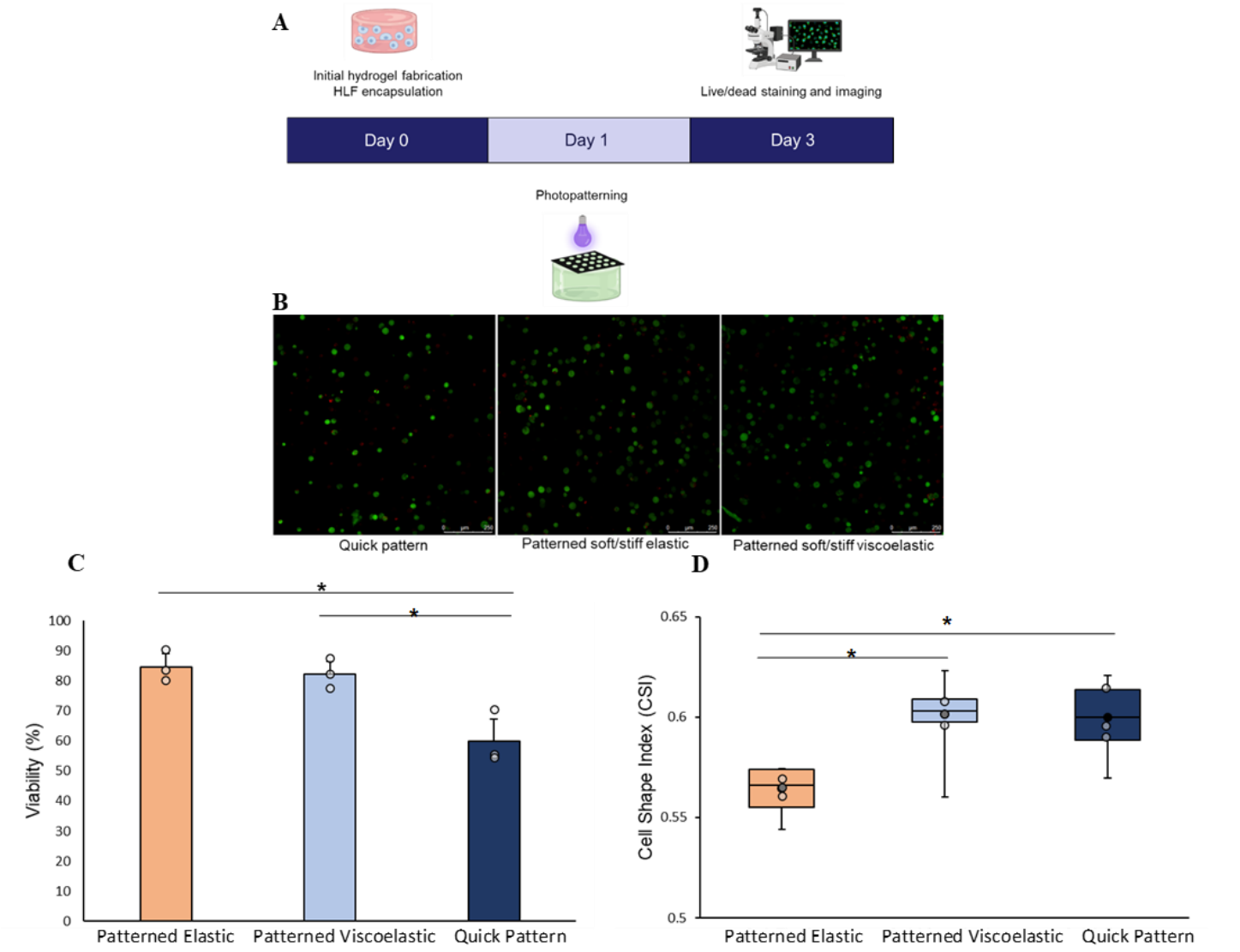

Figure 4. Encapsulation of HLFs over 3-day culture period to assess initial cell behaviors A) Experimental timeline for HLF encapsulation. Initial hydrogel formation and cell encapsulation took place on day 0 with photopatterning occurring on day 1 and imaging occurring on day 3. In quick patterned groups, both the initial encapsulation and hydrogel photopatterning occurred on day 0. **B)** Representative maximum z-projections from each hydrogel group on day 3 of cell culture. Z-stacks are 400 µm thick with images taken every 10 µm and scale bars are 250 µm. **C)** Cell viability for HLFs on day 3 of culture in each hydrogel groups. Both the patterned elastic and viscoelastic groups maintained a high cell viability over the culture period. **D)** Cell spreading over the culture period. HLFs showed more elongation in the patterned elastic groups; however, a longer culture period would need to be conducted to confirm this result. * P<0.005

hydrogel being tested. The softer and stiffer regions of the hydrogel appeared to be about 200 µm apart, which would confirm the efficacy of the photopatterning process in selectively introducing secondary crosslinking to those areas under the transparent portion of the photomasks (**Figure 3**).

HLF encapsulation in 3D hydrogels showed that photopatterning did not negatively impact cell viability

After confirming that the photopatterning system could effectively be applied to 3D hydrogels, HLFs were encapsulated in photopatterned hydrogels to observe initial cell behaviors. Encapsulations took place in hydrogels with

initially soft elastic and initially soft viscoelastic groups. An additional elastic "quick patterned" group was added in which preliminary hydrogel formation and photopatterning took place simultaneously to see if the photopatterning process could be made more efficient.

Encapsulation of HLFs in hydrogels with heterogeneous mechanics was performed by following the photopatterning procedure included in **Figure 3** and adding cells in media to the hydrogel precursor solution. Experiments took place over a 3-day culture period with initial HLF encapsulation occurring on day 0 and photopatterning occurring on day 1 (**Figure 4**). A concern about the photopatterning process was that exposure to the photopatterning solution as well as UV irradiation would negatively impact cell viability; however, quantification of viability after the 3-day culture period showed that a high cell viability was maintained in both patterned elastic and viscoelastic groups (84.6% and 82.3% for elastic and viscoelastic, respectively). Additionally, initial cell metrics suggested increased spreading in hydrogels with elastic mechanics but a longer cell culture experiment would need to be conducted to confirm this result (**Figure 4**).

Discussion

Although IPF is the most common and aggressive form of interstitial lung disease there is currently no cure largely due to a lack of accurate preclinical models that can guide the development of therapeutics. Current models are 2D, non-physiologically stiff, and lack the heterogeneity seen in native tissue. To better model mechanics relevant to IPF, we developed a 3D HA hydrogel with high spatiotemporal control over matrix mechanics so that heterogeneity mimicking the formation of fibrotic nodules seen in IPF progression *in vivo* could be incorporated.[10-13]

The model presented includes many physiologically relevant features including a 3D architecture, degradability through the use of an MMP-degradable crosslinker so that cells can remodel their surrounding ECM, time-dependent mechanics such as viscoelasticity through the use of reversible physical crosslinks, as well as matrix heterogeneity through implementation of photopatterning. The system degradability was first optimized in 3D hydrogels with uniform mechanics. Two MMP-degradable sequences were compared as initial HLF encapsulations showed a lack of cell spreading regardless of hydrogel mechanics as measured by CSI. The two sequences tested were able to be degraded by different HLF-secreted MMPs. With additional cell spreading seen in HLFs encapsulated in hydrogels crosslinked using the VPM degradable peptide sequence, this was the sequence utilized in experiments moving forward.

Building upon previous work in 2D, we successfully implemented photopatterning to introduce heterogeneous mechanics via a light-mediated thiol-ene addition reaction selectively add additional covalent crosslinks to portions of 3D hydrogel models.[8] Hydrogels with an initially low crosslinking density were incubated in a solution containing several components including dithiol crosslinker, LAP photoinitiator, and a thiolate fluorescent peptide for pattern visualization. Confocal microscopy confirmed pattern fidelity through a thickness of 300 μm of the hydrogel.

HLFs, the main mediators of IPF were then encapsulated in hydrogels with heterogeneous mechanics to observe initial cell behaviors relevant to IPF progression and to ensure that viability was not negatively impacted by the photopatterning process. Live/Dead assays confirmed that cell viability remained high through a 3-day culture period. More cell spreading was seen in hydrogels with elastic mechanics; however, additional experimentation over a longer culture period would need to be conducted to confirm this finding.

While this model does incorporate many physiologically relevant components necessary for an effective disease model, it does present limitations. First, cell metrics relevant to fibrosis such as cell spreading would need to be compared between patterned and unpatterned regions of hydrogels to see if behaviors match what is seen *in vivo*. It would be expected that cells would spread more in stiffer portions of the hydrogel; however, previous 3D models have seen behaviors that do not match what has been observed in native tissue. Additional metrics indicative of fibrosis onset should also be measured such as α-smooth muscle actin (α-SMA) stress fiber formation which becomes more prominent in cells during fibrogenesis.

Overall, the ability to incorporate heterogeneous mechanics and viscoelasticity into a 3D hydrogel disease model represents an important step forward in developing an accurate disease model for IPF. Having the ability to model

the disease to identify and test therapeutics would aid in the development of a curative treatment for IPF.

Materials and Methods

NorHA synthesis

Norbornene-modified HA was synthesized in the same manner as in previous experiments conducted in 2D.[8] Briefly, sodium hyaluronate (Lifecore, 74 kDa) was reacted with Dowex 50W proton-exchange resin, filtered, titrated to pH 7.05, frozen, and lyophilized to yield hyaluronic acid tertbutyl ammonium salt (HA-TBA). HA-TBA was then reacted with 5- norbornene-2-methylamine and benzotriazole-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate (BOP) in dimethylsulfoxide (DMSO) for 2 h at 25 °C. The reaction was quenched with cold water, dialyzed (molecular weight cutoff: 6−8 kDa) for 5 days, filtered, dialyzed for 5 more days, frozen, and lyophilized. The degree of modification was 22% as determined by H NMR.

β-CD-HDA Synthesis

The synthesis of β-cyclodextrin hexamethylene diamine (β-CD-HDA) followed the procedure outlined previously.[8] p-Toluenesulfonyl chloride (TosCl) was dissolved in acetonitrile and added dropwise to an aqueous β-cyclodextrin (CD) suspension (5:4 molar ratio of TosCl to CD) at 25 °C. After 2 h, the solution was cooled on ice and an aqueous NaOH solution was added dropwise (3.1:1 molar ratio of NaOH to CD). The solution was reacted for 30 min at 25 °C before adding ammonium chloride to reach a pH of 8.5. The solution was cooled on ice, precipitated using cold water and acetone, and dried overnight. The CD-Tos product was then charged with hexamethylene diamine (HDA) (4 g/g CD-Tos) and dimethylformamide (DMF) (5 mL/g CD-Tos), and the reaction was carried out under nitrogen at 80 °C for 12 h before being precipitated with cold acetone (5 × 50 mL/g CD-Tos), washed with cold diethyl ether (3 × 100 mL), and dried. The degree of modification was 61% as determined by H NMR.

CD-HA Synthesis

β-Cyclodextrin-modified hyaluronic acid (CD-HA) was prepared through coupling of β-CD-HDA to HA-TBA. A reaction containing HA-TBA, 6-(6-aminohexyl)amino-6-deoxy-βcyclodextrin (β-CD-HDA), and BOP in DMSO was carried out at 25 °C for 3 h. The reaction was quenched with cold water, dialyzed for 5 days, filtered, dialyzed for 5 more days, frozen, and lyophilized. The degree of modification was 27% as determined by H NMR.

Peptide Synthesis

All peptides were synthesized using solid-phase peptide synthesis on a Gyros Protein Technologies Tribute peptide synthesizer. A thiolated adamantane (Ad) peptide (Ad-KKKCG), fluorescently labeled peptide (Fluorescein-KKKCG), and MMP-degradable crosslinkers (GCNSVPMSMRGGSNCG and GCNSPQGIWGSNCG) were synthesized on either Rink Amide MBHA high-loaded or Wang resins. Peptides were cleaved, precipitated, then dried overnight.

Cell Encapsulation in HA Hydrogels with Uniform Mechanics

Human lung fibroblasts (HLFs) were used for all experiments. Culture media contained Dulbecco's modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotic antimiotic. For cell encapsulations, all lyophilized polymers were sterilized with germicidal UV irradiation for 2 hours. Elastic hydrogel precursor solutions were created by combining sterilized NorHA (2 wt% NorHA soft, 6 wt% NorHA stiff) with sterile RGD (1 mM), LAP photointiator (1mM), MMP-degradable crosslinker (0.5 thiol-norbornene ratio for soft, 0.6 thiol-norbornene ratio for stiff) and cell media with HLFs at a density of 1 million cells/mL. 50 µL of hydrogel precursor solution was added to cut syringes then irradiated with UV light (365 nm at an intensity of 5 mW/cm^2) for 5 minutes to initiate the thiol-ene addition reaction. Soft (3 wt% NorHA-CD-HA) and stiff (7 wt% NorHA-CD-HA) viscoelastic hydrogels were fabricated by first mixing CD-HA (5 and 8 wt% stock solutions for soft and stiff groups, respectively) with thiolated adamantane peptide at a 1:1 molar ratio to introduce Ad-CD guest-host interactions prior to adding the remaining components. RGD (1mM), MMP-degradable dithiol crosslinker (0.7 thiol-norbornene ratio and 0.8 thiol-norbornene ratio for soft and stiff, respectively), cells in media at a density of 1 million cells/mL and 8 wt% NorHA stock solution were added with LAP photoinitiator (1 mM) before adding 50 µL of solution to cut syringes and irradiating with UV light under the same conditions used to fabricate elastic hydrogels. Hydrogels were stored in cell media with media changed every 2-3 days for 14 days.

Photopatterning HA Hydrogels

NorHA hydrogels (6 wt%) with an initially low crosslinking density (0.3 thiol-norbornene ratio for initially soft elastic and viscoelastic) were fabricated by irradiating with UV light for 4 minutes and swelled overnight in PBS at 37 ºC. Hydrogels were then swelled in a photopatterning solution containing 1 wt% BSA, LAP, MMP-degradable crosslinker, and a thiolated fluorescent peptide for 30 minutes at 37 ºC to yield a final thiol-norbornene ratio of 0.6. Photomasks containing a pattern of alternating transparent and opaque stripes 200 µm thick were then placed over top of hydrogels. Hydrogels were then irradiated with UV light for a second time for 2 minutes before being washed once in PBS.

In hydrogels that were quick patterned, NorHA hydrogels (6 wt%) were fabricated and photopatterned in one step (0.6 thiol-norbornene ratio). 50 µL of hydrogel precursor solution was added to cut syringes then irradiated with UV light for 20 seconds. The photomask was then placed over top of the hydrogel and UV irradiation took place for an additional 5 minutes and 40 seconds before washing in PBS.

Nanoindentation characterization and analysis

Hydrogels were fabricated using the photopatterning process described above then horizontally sectioned. An Optics11 Piuma nanoindenter with a 53.5 µm radius spherical borosilicate glass

probe attached to a cantilever with a spring constant of 4.42 N/m was used for all tests.

An 8 by 8 matrix scan with indentations collected every 50 μm was performed to measure the Young's modulus across 400 μm of the hydrogel in the x-y direction.

The young's modulus was determined by fitting the Hertzian contacts mechanics model to the loading portion of the force vs. distance indentation curves. A Poisson's ratio of 0.5 was assumed.

Cell Encapsulation in photopatterned HA Hydrogels

HLFs were encapsulated in NorHA hydrogels (6 wt%) with an initially low crosslinking density at 1 million cells/mL by mixing cells in media into the hydrogel precursor solution before forming initially soft hydrogels. The same photopatterning process took place as above but hydrogels were returned to cell media for further analysis on cell metrics.

Imaging and Analysis

Live/dead assays were performed using calcein AM (1:2000 dilution) and ethidium homodimer (1:500) to stain for live and dead cells, respectively. Dye solution was added to hydrogels in well plates and incubated for 30 minutes. Hydrogels were imaged on a Leica SP8 confocal microscope and maximum projections were generated from 40 images taken every 10 μm along the z-axis (400 μm in height for each image). Viability was determined by the number of live cells in a given image stack over the total number of live and dead cells and averaged for each gel in a group (n = 3).). Volume (V) and surface area (SA) were calculated via intensity-based cell fluorescence thresholding and the 3D Objects Counter on ImageJ to provide a semi-quantitative analysis approach and to prevent double counting of cells. Cell shape index (CSI), a measure of circularity where a line and a sphere have values of 0 and 1, respectively, was calculated using the formula (V represents volume and SA represents surface area):

$$\boldsymbol{CSI} = \frac{\boldsymbol{\pi^{1/3} 6V^{2/3}}}{\boldsymbol{SA}} \qquad [1]$$

Pattern visualization for photopatterned hydrogels was also conducted via confocal microscopy with the same imaging parameters.

Statistical Analysis

One-way ANOVA with Tukey's HSD post hoc tests were performed for all quantitative data sets. All experiments included at least 3 hydrogels and/or at least 20 individual cells per group.

End Matter

Author Contributions and Notes

S.R.C., E.H., and K.G. designed research, E.H. and K.G. performed research, K.G. and E.H. analyzed data; and K.G. wrote the report.

The authors declare no conflict of interest.

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