Published as: *Biomaterials*. 2015 May ; 49: 47–56.

Dynamic stiffening of poly(ethylene glycol)-based hydrogels to direct valvular interstitial cell phenotype in a three-dimensional environment

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Keywords

heart valve; valvular interstitial cells; hydrogel; elasticity; three-dimensional cell culture; ECM

1. Introduction

Valvular interstitial cells (VICs) are the most prevalent cell type in heart valves and actively regulate the progression of valve disease (1). In a healthy valve, the majority of VICs are quiescent fibroblasts (2). These cells can be activated to myofibroblasts, which exhibit increased proliferation, cytokine secretion, and matrix remodeling, and are associated with disease progression (1,3–5). The fraction of the population of VICs exhibiting the myofibroblast phenotype changes throughout a person's development and lifetime. Early in development, the majority of VICs are positive for α SMA. In healthy adults, almost all of the VICs are quiescent, but activation levels are increased once again in diseased valves (6).

VIC phenotype has been characterized using various metrics. Alpha smooth muscle actin (α SMA) is a commonly used marker for myofibroblast-like cells (7,8). The presence of organized α SMA stress fibers as determined by immunostaining is often used to classify VICs as quiescent fibroblasts (no stress fibers) or activated myofibroblasts (stress fibers present) (3,9,10). In contrast, the mesenchymal marker S100A4 (also known as fibroblast-specific protein 1) has been shown to increase when VICs are quiescent (11). These measures provide useful readouts for a general screen to determine how VIC phenotype responds to various treatments or culture conditions.

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With respect to the VIC microenvironment, the matrix mechanics can be an important regulator of VIC phenotype, where elastic modulus has been shown to influence VIC myofibroblast activation. While variations exist in the values reported for the threshold for activation, 2D experiments generally show that lower substrate modulus ($< \sim 5$ kPa) leads to mostly quiescent VICs and higher substrate modulus ($> \sim 25$ kPa) activates most VICs to myofibroblasts, with a range of activation levels observed at intermediate moduli (9,10). These trends recapitulate aspects of valve disease, with the stiffer, disease-like substrates leading to the myofibroblast phenotype that is more prevalent in diseased valves. Additionally, this activation is reversible and responsive to changes in the local environmental mechanics, where *in situ* softening of hydrogels has been used to study VIC deactivation (12). Substrate modulus has also been shown to influence VIC morphology and calcification, with stiffer substrates leading to a more spread, elongated morphology and higher levels of calcium deposition (10,13). While there has been much progress in understanding how VICs respond to mechanical and biochemical cues in two dimensions, less is known about how these factors may influence phenotype in a three-dimensional environment.

The dimensionality of a cell's microenvironment can profoundly impact function (e.g., proliferation, morphology, polarity, motility), and changes in dimensionality can limit cell-cell interactions, availability of soluble factors (14), and even influence gene expression (15). Butcher *et al.* encapsulated VICs in collagen gels and found that encapsulated VICs (3D) expressed less α SMA compared to VICs seeded on top of collagen gels (2D) (16). As a complement to collagen and other naturally-derived protein matrices, Benton *et al.* encapsulated VICs within proteinase-degradable, PEG-based hydrogels and found that when the adhesive peptide, RGDS, was incorporated, the cells attached to the matrix via $\alpha_v\beta_3$ integrins and were able to spread and elongate within the hydrogel (17). Furthermore, α SMA expression was found to increase with culture time over 14 days and was dependent on TGF- β 1 treatment. These studies have improved our understanding of how VICs behave in response to 3D culture, and have motivated the study of the influence of matrix mechanics on VIC phenotype in 3D.

To elucidate cellular response to mechanical cues in 3D, VICs have been co-encapsulated with PEG microrods of varying moduli within Matrigel; VICs exposed to stiff microrods exhibited reduced α SMA production and decreased proliferation (18). This study showed a relationship between the presence of stiff microrods and myofibroblast deactivation, but provided mechanical differences by the use of discrete regions of higher modulus rather than changing the modulus in the entire volume to which the cells were exposed. To more directly measure forces exerted by encapsulated cells, VICs have been encapsulated in fibrin gels attached to posts providing a range of boundary stiffnesses, where it was observed that the combination of stiff boundary posts and addition of TGF- β 1 resulted in increased cell force generation (19). Duan *et al.* encapsulated VICs within hyaluronic acid-based hydrogels to study their response to modulus in a 3D environment. Hydrogel modulus was varied by changing the hyaluronic acid molecular weight and degree of methacrylation and by incorporating methacrylated gelatin into the hydrogels. By immunostaining, they demonstrated an increased number of α SMA-positive VICs in softer hydrogels (8).

Although VICs were found to be more myofibroblast-like in the lower-modulus environment, interpretation of these results is somewhat confounded by the coupling of cell morphology with the density of the surrounding matrix.

In general, a significant obstacle to studying cellular responses to matrix mechanical properties in 3D is separating highly coupled variables. For example, when cells are encapsulated in a matrix metalloproteinase (MMP)-degradable synthetic hydrogel or natural gel (e.g., collagen, Matrigel, hyaluronan), the cells are able to spread and elongate because they locally remodel their environment. This remodeling often means softening of the local gel, and a complex coupling of cell shape and local material properties. In other words, it can be difficult, at best, to independently control local gel chemistry, mechanics, and cellular interactions/morphologies, and while advances in light microscopy allow detailed characterization of real time changes in cell functions, it can be more difficult to similarly characterize real time changes in gel properties.

To address some of this complexity, materials with dynamic control of the cell microenvironment can help de-convolute some of these variables. For example, Burdick and co-workers used a hydrogel platform with staged crosslinking to create interpenetrating networks of hydrogels with varying degradability and the ability to increase modulus (i.e., stiffen) after initial gel formation (20). They demonstrated the temporal effects of a modulus increase on mesenchymal stem cells in 2D where cells on stiffened substrates had larger cell area and exerted greater traction forces (21). This system was also adapted for 3D experiments to show that the formation of a secondary, non-degradable network around encapsulated mesenchymal stem cells directed the cells towards adipogenesis, while cells encapsulated in gels that did not undergo secondary crosslinking favored osteogenesis (22).

Building on this concept, we use PEG-based hydrogels formed via a photochemical thiol-ene polymerization to study the influence of matrix modulus on VIC activation in 3D environments. VICs were encapsulated within MMP-degradable, PEG-based hydrogels of varying moduli. VIC phenotype was assessed by quantitative real-time polymerase chain reaction (qRT-PCR) and by immunostaining for α SMA. To control for differences in cell morphology that typically arise when encapsulating cells in hydrogels with varying crosslinking density, we developed a cytocompatible *in situ* stiffening system. VICs encapsulated in low-modulus gels were allowed to spread and elongate, and then stoichiometric amounts of an 8-arm PEG-norbornene and an 8-arm PEG-thiol along with a photoinitiator were diffused into the gel. Photopolymerization of the cell-laden gel containing the stiffening solution increased the modulus of the gel without compromising cell viability. These dynamically stiffening gels give the experimenter control of the local environment surrounding the cells in three dimensions without altering cell morphology.

2. Materials and Methods

2.1. Synthesis of poly(ethylene glycol)-norbornene (PEGnb)

PEG-norbornene was synthesized using a previously described protocol (23). Briefly, equimolar amounts of 8-arm PEG (JenKem) with a molecular weight of either 10 kDa or 40 kDa and 4-dimethylaminopyridine (Sigma-Aldrich) were dissolved in a minimal amount of

anhydrous dichloromethane (Sigma-Aldrich), under argon in a round bottom flask. 16 equivalents each of 5-norbornene-2-carboxylic acid (Sigma-Aldrich) and N,N'-diisopropylcarbodiimide (Sigma-Aldrich) were added and reacted overnight on ice. Product was precipitated in 4°C ethyl ether (Fisher Scientific), filtered, and then dried by vacuum. PEG-norbornene was purified by dialysis and lyophilized. Greater than 90% functionalization was achieved as determined by proton nuclear magnetic resonance imaging and rheological characterization of the crosslinked product.

2.2. VIC isolation and culture

VICs were isolated from fresh porcine hearts (Hormel) as previously described (24). Aortic valve leaflets were removed and rinsed in a wash solution containing Earle's Balanced Salt Solution (Life Technologies) with 1% penicillin-streptomycin (Life Technologies) and 0.5 µg/mL fungizone (Life Technologies). Then, the leaflets were incubated in 250 units/mL collagenase (Worthington) solution for 30 min. at 37°C and vortexed to remove endothelial cells. Cells were then incubated with collagenase solution for 60 min. at 37°C and vortexed. This solution was filtered with a 100 µm cell strainer and centrifuged. The cell pellet was resuspended in growth media consisting of Media 199 with 15% fetal bovine serum (FBS, Life Technologies), 2% penicillin-streptomycin (Life Technologies), and 0.5 µg/mL fungizone (Life Technologies). Cells were cultured at 37°C and 5% CO₂ on tissue culture polystyrene (TCPS) for expansion before experiments. Cells were passaged using trypsin (Life Technologies) digestion. For experiments, media serum level was reduced to 10% FBS to reduce proliferation.

2.3. Hydrogel formation and characterization

VICs were encapsulated within MMP-degradable, PEG-based hydrogels using a photoinitiated thiol-ene reaction (25). Eight-arm PEG-norbornene was reacted with the peptide crosslinker KCGPQG↓IWGQCK (American Peptide Company, Inc.) and 2 mM CRGDS adhesive peptide (American Peptide Company, Inc.) in serum-free, phenol red-free media (Life Technologies) using the photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP, 1.7 mM) (Figure 1). Cells in suspension were added to the gel precursor solution for a final concentration of 10 million cells/mL. 29 µL of gel solution was added to a 6 mm rubber mold on top of a glass slide treated with SigmaCote (Sigma-Aldrich) to prevent attachment of the gel to the slide. The solution was polymerized under UV light centered around 365 nm at 2 mW/cm² for 3 minutes. The shear modulus of the swollen hydrogels was measured using an Ares 4400 rheometer (TA Instruments) with a parallel plate geometry. Both frequency sweeps and strain sweeps were performed to ensure that measurements were representative of the linear viscoelastic regime. The shear modulus (G') was measured and converted to Young's modulus (elastic modulus, E) using the formula $E = 2G(1+\nu)$ with $\nu = 0.5$ (26). The loss modulus is much lower than the storage modulus ($G'' \ll G'$) for all gel conditions, indicating that these gels primarily exhibit elastic behavior (data not shown).

2.4. Hydrogel stiffening and characterization

Stiffening solutions were comprised of 8-arm 10 kDa PEG-norbornene (2 or 10 mM), 8-arm 10 kDa PEG-thiol (2 or 10 mM), and LAP (2.5 mM). For control gels, the PEG-thiol was

replaced with cysteine (Sigma-Aldrich) to create a reacting formulation that did not form a gel. Gels were soaked in the stiffening solution for 12 minutes. Gels were then removed from solution and the excess swelling solution was blotted from the surface before polymerization with UV light (2 mW/cm^2 at 365 nm) for 3 minutes. The shear modulus after stiffening was measured as described for the initial gel formation.

2.4 Cell viability and morphology analysis

Cell viability was measured using a Live/Dead cytotoxicity kit (Life Technologies), which is a membrane integrity assay. Cell-laden hydrogels were incubated in PBS containing $1 \mu\text{M}$ calcein and $4 \mu\text{M}$ ethidium homodimer for 30 minutes. Cells were imaged on a 710 LSM NLO confocal microscope (Zeiss) and maximum intensity projections were created from 11 images each $10 \mu\text{m}$ apart for a total height of $100 \mu\text{m}$. Fraction of live cells was quantified by manual counting. Morphology measurements were made using ImageJ to measure the length and width of cells based on calcein (live) staining.

2.4. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Messenger RNA (mRNA) was isolated from VICs using TriReagent (Sigma-Aldrich) with isopropanol (Sigma-Aldrich) extraction and ethanol (Sigma-Aldrich) washes according to the manufacturer's instructions. Reverse transcription was performed using the iScript cDNA Synthesis kit (Bio-Rad) and the Eppendorf Mastercycler. qRT-PCR was performed using an iCycler machine and iQ SYBR Green Supermix (Bio-Rad) with custom primers (Table 1, Illumina) at 300 nM . Each well also contained $0.05 \text{ ng}/\mu\text{L}$ of the cDNA. Values were calculated by comparing each sample to a standard curve, and data was normalized to an internal standard, L30. αSMA , S100A4, CTGF, COL1A1, MMP1, and L30 expression levels were measured.

2.5. Immunostaining for αSMA and f-actin

Cells were fixed with 10% formalin (Sigma-Aldrich). Samples were then washed with PBS and permeabilized with 0.05% TritonX100 (Fisher Scientific) in PBS. Non-specific staining was blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS with Tween20 (Sigma-Aldrich) and then incubated with mouse anti- αSMA (Abcam) overnight at 4°C . Samples were then washed and incubated with the secondary antibody, goat-anti-mouse AlexaFluor 488 (Life Technologies) and TRITC-phalloidin (Sigma-Aldrich). Next, cells were treated with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies) to stain the cell nuclei. Cells were imaged on a 710 LSM NLO confocal microscope (Zeiss). Myofibroblast percentages were quantified by manually counting cells expressing organized αSMA stress fibers.

2.6. Statistical Analysis

For each experiment, at least 3 biological replicates were included. Each biological replicate consisted of cells pooled from separate groups of porcine hearts. For qRT-PCR, each biological replicate is an average of at least 3 technical replicates. For morphology analysis, at least 25 cells were analyzed per condition per replicate. At least 100 cells per condition per replicate were analyzed for cell viability and immunostaining experiments. Data were

compared using one-way ANOVAs and Tukey post-tests in Prism 5 (GraphPad Software, Inc). Data is presented as mean $\pm$ standard error.

3. Results

3.1. VIC response to matrix mechanics in 3D

To study VIC response to stiffness in 3D, cells were first encapsulated in peptide-functionalized PEG hydrogels of varying moduli (Figure 1). A range of moduli from 0.24 kPa to 12 kPa was achieved by varying the molecular weight and concentration of 8-arm PEGnb while controlling the ratio of –ene groups on the PEGnb to thiol groups on the peptides. These gels were crosslinked using a peptide (KCGPQG↓IWGQCK) that can be cleaved by MMPs secreted by VICs (17). The fibronectin-derived adhesive peptide CRGDS was incorporated at 2 mM to facilitate cell adhesion to the gel. There was a dependence on overall cell viability on hydrogel modulus, from ~90% viability in 0.24 kPa gels to ~60% viability in 12 kPa gels after 2 days (Figure 2B). This decrease in viability with matrix stiffness may be due to factors related to cell-matrix interactions and overall morphology and their influence on apoptosis. Here, only VICs in the 0.24 kPa gel were able to spread and elongate after 2 days, while VICs encapsulated in the 4 kPa or 12 kPa gels remained smaller and rounded (Figure 2A). mRNA levels were measured using qRT-PCR to characterize phenotypic differences over the range of gel moduli. There was an inverse relationship between gel modulus and α SMA mRNA expression in this system, with the cells in the 12 kPa gel containing only ~20% as much α SMA mRNA as the 0.24 kPa gels (Figure 2C). Immunostaining showed activated myofibroblasts with α SMA stress fibers only in the 0.24 kPa gels, while the 4 kPa and 12 kPa did not exhibit organized α SMA or f-actin fibers (Supplemental Figure 1). Based on this data alone, it was unclear whether this observation was due to a direct response to gel modulus or a result of differences in cell viability or morphology.

3.2. Dynamic stiffening of cell-laden hydrogels

To decouple the effects of morphology versus matrix mechanics on VIC activation, an approach to stiffen the hydrogel modulus *in situ* was pursued. VICs were first encapsulated within the low-modulus (0.24 kPa) hydrogel, where the low crosslinking density facilitated timely cell spreading. Encapsulated cells were allowed 3 days to locally remodel the gels and spread and elongate within the gels post-encapsulation. Then, the gels were stiffened by swelling in solutions containing equal amounts of 8-arm PEGnb and 8-arm PEG-thiol, as well as the photoinitiator, LAP, that were subsequently photopolymerized *in situ*. For the soft “to medium” condition, PEGnb (2 mM) and PEG-thiol (2 mM) were added. For the soft “to stiff” condition, the concentrations of PEGnb and PEG-thiol were both increased to 10 mM. Using this approach, a greater than 50-fold increase in modulus was achieved (Figure 3). The final gel modulus was dependent on the concentration of PEG in the stiffening solution, with the “to medium” solution increasing the modulus from 0.24 kPa to 1.2 kPa and the “to stiff” solution increasing the modulus to 13 kPa. By stiffening, the cell-defined matrix is transformed to a more user-defined, non-degradable environment in which the cells are unable to elicit significant changes in the local modulus. Cell viability was measured 2 days post-stiffening by live/dead staining (Figure 4B). Here, we note high levels of survival,

indicating a stronger effect of VIC spreading and morphology on viability compared to matrix modulus. Furthermore, VIC morphology was characterized 2 days after stiffening by quantifying the average cell aspect ratio (cell length/cell width) in each of the conditions (Figure 4C). Note that VICs maintained a similar morphology across the entire range of moduli studied, with the average cell aspect ratio in stiffened gels only ~30% lower than in soft gels. For comparison, the differences in the aspect ratio of cell directly encapsulated in the 0.24 kPa, 4 kPa, and 12 kPa hydrogels (Figure 3) varied by as much as 70%. Thus, the similar morphology in the stiffening system across the range of moduli studied aided in decoupling gel modulus and cell morphology, two frequently paired variables.

3.3. Hydrogel modulus directs VIC phenotype in 3D

Using this *in situ* stiffening approach, we found that α SMA mRNA levels decreased with stiffening. VICs in gels stiffened to 13 kPa exhibited only $23 \pm 2\%$ as much α SMA mRNA as VICs in the 0.24 kPa gels (Figure 5). This trend was confirmed by immunostaining for α SMA stress fibers (Figure 5A). In the soft gels, $42 \pm 6\%$ of VICs exhibited organized stress fibers containing α SMA, a hallmark of the myofibroblast phenotype. Only $13 \pm 3\%$ of VICs in gels stiffened to 1.2 kPa exhibited α SMA stress fibers; however, some diffuse α SMA staining was also visible. VICs in gels stiffened to 13 kPa displayed very little α SMA in either form ($2.5 \pm 0.1\%$ activation) (Figure 5B). To ensure that these phenotypic changes were not due to exposure to the swelling solution, radical intermediates, or UV light, the stiffening process was performed on VICs encapsulated in soft gels, but the 8-arm PEG-thiol was replaced with cysteine to make a reacting stiffening solution that would not form a gel. We found that the stiffening process did not lead to any statistically significant differences in activation levels (Figure 5C).

Further, evidence for the deactivation of VICs upon matrix stiffening was collected by analyzing gene expression. Here, α SMA mRNA levels decreased with increasing stiffness. The myofibroblast marker CTGF also showed a downward trend with stiffening (Figure 6). In contrast, increasing stiffness led to an increase in mRNA levels of S100A4, which has been correlated to decreases in activation and α SMA expression in VICs and in other cells types (11,27,28). Genes associated with matrix remodeling were also investigated. While a greater than 3-fold increase in MMP1 mRNA expression was seen in each of the stiffened conditions compared to the VICs in the soft gels, expression of the collagen type I precursor Col1A1 remained relatively unchanged by stiffening at the time point examined in this experiment (Figure 6). Together, these data indicate that VICs cultured in 0.24 kPa hydrogels tend towards a myofibroblast-like phenotype, with a spread morphology and strong matrix interactions. In contrast, if the surrounding gel environment is stiffened *in situ*, VICs appeared to revert to a more quiescent fibroblast phenotype. We hypothesize that this phenotypic change is due to changes in the modulus of the extracellular environment and is not caused by differences in morphology. While an elongated morphology is likely required for activation to the myofibroblast phenotype, this morphology alone does not appear to direct cells to the activated state.

4. Discussion

Many factors influence how cells respond to cues from their local microenvironment. Matrix density, adhesivity, and elastic modulus can all affect cell phenotype directly, but also influence variables such as cell shape. Generally speaking, cells sense the ECM through focal adhesions that can involve different integrins and proteins, further complicating the understanding of this outside-in signaling. Many of these matrix interactions can be isolated and studied on 2D surfaces, and these experiments have helped define our understanding of the role of the extracellular space in directing and maintaining cell function. However, in other cases, understanding of cellular responses *in vivo* may benefit from more advanced culture systems and measurements in 3D systems. In this work, we were interested in better understanding how mechanical signaling from the extracellular matrix can contribute to VIC function.

VICs are key regulators in valve repair and disease, which involve dynamic changes in the extracellular environment with time. Here, we sought to exploit some unique material chemistry to show how VICs and their myofibroblast properties might respond to dynamic changes in stiffness in a 3D environment. Specifically, these experiments employed a peptide-functionalized hydrogel synthesized with thiol-ene chemistry to create proteolytically degradable hydrogels (Figure 1). These gels were then subsequently stiffened on demand using a secondary, non-degradable network (Figure 3). One significant advantage of this system is the ability to study the impact of matrix stiffness on cells while keeping a similar morphology across a large range of elastic moduli that are relevant to valve tissue properties. Additionally, the use of a non-degradable stiffening network provides more control over the local modulus experienced by the cells throughout the course of the experiment compared to a proteolytically degradable network.

In most hydrogel culture systems, stiffer gels arise from networks with a higher density of crosslinks or polymer content, so this necessitates that cells cleave more crosslinks to achieve a spread morphology similar to those observed in softer gels with lower crosslinking density. This coupling of morphology to network density makes it difficult to deconvolute the effects of cell morphology from matrix stiffness, where cell shape can correlate to underlying matrix adhesion and density. The ability to independently study morphology and stiffness is of significant interest because of the many ways in which cell morphology can direct cell phenotype (29,30). Here, we follow an approach by Khetan *et al.* to attribute phenotypic changes in VICs directly to changes in modulus by eliminating morphology as a variable through *in situ* stiffening of soft gels (20).

Specific to the VICs, an advantage of the stiffening platform was the high cell viability achieved across the entire range of moduli studied, compared to decreasing viability observed when VICs are directly encapsulated in stiff gels and restricted to a rounded morphology for extended culture times (Figures 2, 4). Differences in cell viability are important not only because of changes in cell density, but also because there is a possibility that one might inadvertently select for a specific population of VICs. The latter can be especially problematic for primary VICs, which represent a heterogeneous population of cells isolated from valve tissue (31).

The specific hydrogel moduli evaluated as part of this study correspond to moduli of relevance to aortic valves. The 0.24 kPa gels are softer than adult valves and might be viewed as corresponding more closely to a developing valve. The 1.2 kPa gels approach the moduli measured in healthy adult valves, which has been reported to range from 1.1 kPa to 1.6 kPa for the ventricularis and fibrosa valve layers, respectively (32). Finally, the 12 kPa gels were chosen to provide a higher modulus condition, as matrix stiffening occurs in diseased valves. While 12 kPa is not an upper limit or dividing threshold for myofibroblast activation or the onset of valve fibrosis, it is stiffer than what might be expected in a healthy valve.

Early efforts to study VIC-matrix interactions and mechanotransduction have observed that seeding VICs on softer (~7 kPa) hydrogel substrates better preserves their native phenotype compared to culture on tissue culture polystyrene (TCPS) (33). This study identified thousands of genes that were differentially regulated on TCPS compared to freshly isolated cells and found that three of the genes upregulated on TCPS (α SMA, CTGF, and Col1A1) were expressed at levels closer to freshly isolated cells when the VICs were instead cultured on the soft hydrogel. Additionally, the percentage of VICs seeded on soft gels exhibiting α SMA stress fibers was similar to the percentage found in the valve, while much higher levels of activation were observed on TCPS. To better understand the role of substrate mechanics in regulating these profound differences that were observed with culture conditions, VIC response to dynamic changes in substrate modulus was also studied; specifically, VICs were seeded on a hydrogel with photodegradable moieties to allow *in situ* softening. These experiments revealed that VIC activation on stiff substrates is reversible, and the myofibroblast phenotype can be deactivated by a reduction in the substrate modulus (12). While softening systems have been exploited to study the response of VICs to a dynamic softening of the matrix environment, there are few examples of studying how *increases* in the matrix modulus influence cell phenotype *in situ*. Studying cellular responses to increases in modulus is important because many *in vivo* processes involve increases in tissue stiffness. For example, increases in tissue stiffness are frequently associated with fetal development, the progression of fibrotic diseases, and cancerous tumors (34–36). As a result, there is a growing interest in biomaterials scaffolds for 3D cell culture that enable one to stiffen the matrix mechanics on demand. In this regard, one system that allows *in situ* modulation of gel mechanics was developed by the Burdick group to study mesenchymal stem cell fate (37). The initial hydrogels were formed using a Michael addition of acrylated hyaluronic acid with cysteine-containing MMP-degradable peptides where the acrylate functionality was in excess. Later, gels were stiffened using a photoinitiated radical polymerization of the remaining acrylate groups. By using a photoinitiated reaction for the secondary crosslinking, they were able to avoid stimuli that are known to elicit cellular responses, such as pH or temperature. Here, we develop a complementary approach using a peptide functionalized PEG-based system that allows the formation of highly defined step-growth networks with tailored presentation of peptide signaling moieties, along with highly defined control of biomechanics and biodegradation. These material characteristics are important to control for VIC cultures, and PEG can provide an alternative to hyaluronic acid, which can influence VIC phenotype upon degradation into lower molecular weight fragments (38). Finally, the thiol-ene reaction mechanism has been shown to be more

cytocompatible for some highly sensitive primary cell types, and also allows tethering of many growth factors without a loss in activity (39–41).

Perhaps most unexpectedly, trends in VIC phenotype (e.g., myofibroblast activation) with stiffness in 3D environment were quite different from those observed in 2D cultures. Specifically, VIC activation typically correlates with stiffness of the culture substrate in 2D experiments (9,12), and this observation is consistent with clinical reports of higher levels of persistent myofibroblasts during fibrotic valve disease progression. However, less is known about how myofibroblast properties correlate with VIC cultures in 3D, and this can be important for tissue engineers seeking to regenerate functional valve leaflets or for biochemists seeking to identify small molecule inhibitors that might reverse valve disease. When encapsulated in hydrogel environments, VICs respond to the stiffness of their environment in a manner that one might expect for a well-functioning valve: in an initially soft matrix in early stages of development or in valve tissue that has been damaged after injury, VICs become activated myofibroblasts and deposit additional extracellular matrix proteins. When the matrix surrounding the cells has reached an appropriate density and stiffness, VICs return to a quiescent phenotype, preventing the pathological state of continued activation and tissue deposition. In the dynamic gel conditions examined here, where VICs were encapsulated in soft gels, allowed to spread, and then *in situ* stiffened, VICs residing in the stiffest gels were nearly all quiescent fibroblasts (Figure 5). These experiments supported the hypothesis that stiffness alone does not activate VICs to their myofibroblast phenotype and that additional stimuli may be necessary to promote their persistent activation, whether for healthy tissue remodeling or misregulation that is related to disease progression. The observations of the dependence of VIC activation on matrix modulus are also consistent with aspects of fetal development, where the soft valve matrix leads to high percentages of activated myofibroblasts, but as the valve develops and stiffens, the percentage of myofibroblasts decreases (6,42). Similar levels of deactivation are seen after stiffening VIC-laden hydrogels with either degradable or non-degradable networks, providing additional evidence to support the role of modulus in deactivation (Supplementary Figure 2). It would be interesting to expand the range of bioscaffold elasticities and the timing of the stiffening to see if certain culture conditions are more reminiscent of valve development versus disease progression.

Beyond examining α SMA protein expression and organization, the VIC phenotype was further characterized in these 3D gel environments using qRT-PCR to examine mRNA levels of myofibroblast markers, fibroblast markers, and matrix remodeling genes (Figure 6). Consistent with the immunostaining results, α SMA mRNA levels were highest for VICs encapsulated in the softest gels, and increasing gel stiffness correlated with decreasing α SMA expression. CTGF, another myofibroblast marker, was also examined. While there was greater variation seen in the CTGF mRNA levels, a general trend of decreasing CTGF was observed. The lower expression of both of these myofibroblast markers in the stiffened gels is consistent with the lower activation levels seen by immunostaining. S100A4, an indicator of the quiescent fibroblast phenotype, was also examined. S100A4 increased with increasing stiffness, suggesting that VICs were deactivated to the fibroblast phenotype upon stiffening. Collectively, the changes in expression of these three markers are consistent with

the immunostaining data (Figure 5), which showed more myofibroblasts in the soft gels than in the stiffened gels.

One of the major differences between 2D and 3D hydrogel cultures is that encapsulated cells must degrade and remodel their surrounding environment to spread. As a result, one might expect variations in MMP activity as a function of dimensionality, and increased matrix deposition and MMP activity are typically associated with VIC myofibroblasts. For example, MMP1 was found to be about 17 times as prevalent in stenotic human aortic valves compared to healthy valves, while a 23 fold increase was reported in COL1A1 mRNA, a precursor of type I collagen (43). Interestingly, we observed that MMP1 mRNA expression was higher in the stiffer gels, where almost all VICs are quiescent (Figure 6). This increased expression with stiffening may be a result of the degradability of the gels. With the stiffening approach, the cells become constrained by the non-degradable network and may express more MMPs in an attempt to modify their local environment. In contrast, the VICs remaining in degradable, soft gels may be able to cleave crosslinks even with lower MMP expression. This explanation is consistent with the nearly equal levels of MMP1 mRNA in both of the stiffened conditions (“to medium” and “to stiff”), but it would be interesting to adjust the stiffening formulation to also study a proteolytically degradable system. However, interpretation of such experiments would be confounded by local changes that would occur in matrix mechanics with different degradation rates of the loosely and densely crosslinked formulations. Finally, no statistically significant differences in COL1A1 were observed in this study across the conditions (Figure 6), but it is possible that changes in COL1A1 would be observed on a longer timescale than the other markers that were examined here. Even if no differences are measurable at the mRNA level, long-term studies of the deposition of collagen within the gels should provide more insight as to how modulus and MMP activity may direct matrix remodeling in 3D. Here, we intentionally selected earlier time points to test hypotheses related to VIC response to changes in the synthetic ECM matrix, before they significantly alter their microenvironment through significant ECM deposition.

In summary, peptide-functionalized hydrogels formed through thiol-ene photopolymerizations provide a versatile platform for 3D culture of VICs. Through sequential reaction steps, this platform enabled us to transition from simple, cellularly-remodeled materials to a user-defined system with *in situ* tunable mechanical properties. While we only showed the effect of a single stiffening event on VIC phenotype, it would be possible to repeat the stiffening procedure multiple times to simulate the more gradual stiffening that would occur *in vivo*. It might be particularly interesting to repeatedly stiffen with degradable gel formulations and couple these experiments with real time measurements of VIC functions and matrix remodeling (e.g., traction force microscopy).

5. Conclusions

In summary, we developed an approach for *in situ* stiffening of cell-laden hydrogels using thiol-ene chemistry, and the synthetic methods were implemented to study VIC phenotype in 3D environments. VICs encapsulated within soft ($E = 0.24$ kPa) gels had an elongated morphology and high levels of expression of the α SMA myofibroblast marker, while VICs

encapsulated within higher modulus ($E = 4\text{--}12$ kPa) gels were mostly rounded and had low levels of α SMA expression. To delineate morphological effects from modulus effects, an *in situ* gel stiffening approach was pursued. In 0.24 kPa gels, ~40% of VICs were activated to the myofibroblast phenotype, but when stiffened to 13 kPa, only ~2% of VICs remained activated 2 days after the stiffening. This response was also evaluated at the mRNA level, where more than a 70% reduction in α SMA levels was observed. This trend of reduced activation with increasing microenvironmental stiffness is counter to observations when VICs are cultured on 2D hydrogel surfaces, and implies that factors associated with dimensionality may play an important role in better understanding VIC phenotypic changes, especially the myofibroblast-to-fibroblast transition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Samuel Payne for help with VIC cultures and Dr. Huan Wang for PCR primers. The authors would also like to acknowledge support to KMM from an NIH Pharmaceutical Biotechnology Training Grant, funding from an NIH grant (R01 HL089260) and HHMI.

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Figure 1. Schematic of VIC encapsulation within PEG-based hydrogels. PEGnb average molecular weight and concentration of PEGnb and MMP-degradable crosslinking peptide were varied as shown in the table to achieve a range of moduli. VICs were encapsulated at a density of 10 million cells per mL of gel. Insets (50 μ m) show Live/Dead staining at day 0 and day 2.

Figure 2.

A. Live/Dead staining of VICs 48 hours after encapsulation within hydrogels. VICs within 0.24 kPa hydrogels exhibit an elongated morphology and high cell viability, while VICs within 4 kPa and 12 kPa retained a rounded morphology and experienced greater cell death. Scale bar = 100 μm. B. Quantification of Live/Dead staining shows decreased cell viability with increasing modulus. C. Increasing modulus correlates with decreasing αSMA mRNA relative to the L30 internal standard as determined by qRT-PCR. * = $p < 0.05$.

Figure 3.

Schematic of stiffening the cell-laden hydrogels. 8-arm PEG-thiol, 8-arm PEGnb, and LAP were swollen into the cell-laden gels for 12 minutes. Gels were then re-polymerized using UV light to increase the gel modulus. Final stiffened modulus was varied by changing the concentration of PEG-thiol and PEGnb while maintaining a stoichiometric reaction.

Figure 4.

A. Live/Dead staining of VICs encapsulated within soft and stiffened hydrogels 48 hours after stiffening. VICs in all conditions exhibited an elongated morphology and high cell viability. Scale bar = 100 μ m. B. Quantification of Live/Dead staining shows high cell viability across the range of moduli. C. Quantification of cell aspect ratio. High modulus standard encapsulations result in greatly reduced cell aspect ratio, while aspect ratio in stiffened gels remains approximately the same as in the softest condition. * = $p < 0.05$.

Figure 5.

A. Immunostaining for α SMA (green), f-actin (red) and nuclei (blue) with two representative images per condition. In soft gels, many cells exhibit α SMA stress fibers. In “to medium” gels, there are fewer cells positive for stress fibers, but diffuse α SMA is common. In “to stiff” gels, very little α SMA is present in either form. Scale bars = 100 μ m. B. Fraction of activated VICs as defined by the presence of α SMA stress fibers was quantified. Activation decreased with increasing final modulus. C. A non-gelling formulation of the stiffening solution was used as a control to show that the UV light, radical intermediates, and other possible strains of the stiffening process were not responsible for the reduction in activation. * = $p < 0.05$.

Figure 6.

qRT-PCR comparing genes of interest in soft (white), to medium (striped), and to stiff (solid) conditions. All genes were normalized to the L30 housekeeping gene. Myofibroblast markers αSMA and CTGF decreased with increasing modulus. The fibroblast marker S100A4 increased with increasing modulus. MMP was higher in the stiffened gels than in the soft gel. Col1A1 was unchanged in the examined conditions. * = $p < 0.05$.

Table 1

Primer sequences for qRT-PCR.

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
L30	GCTGGGGTACAAGCAGACTC	AGATTTCCTCAAGGCTGGGC
α SMA	GCAAACAGGAATACGATGAAGCC	AACACATAGGTAACGAGTCAGAGC
S100A4	GAGCTAAAGGAGTTGCTGACC	CTGTCCAGGTTGCTCATCAG
CTGF	CTGGTCCAGACCACAGAGTGG	GCAGAAAGCGTTGTCATTGG
MMP1	GGCATCCAGGCCATCTATG	CACTTGTGGGTTTGTGGG
COL1A1	GGGCAAGACAGTGATTGAATACA	GGATGGAGGGAGTTTACAGGAA