Biomaterials 49 (2015) 47–56

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

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

Kelly M. Mabry [a], Rosa L. Lawrence [b], Kristi S. Anseth [a, c, *]

[a] Department of Chemical and Biological Engineering, University of Colorado at Boulder, Boulder, CO 80309, USA
[b] Fairview High School, Boulder, CO 80305, USA
[c] Howard Hughes Medical Institute and the BioFrontiers Institute, University of Colorado at Boulder, Boulder, CO 80309, USA

ARTICLE INFO

*Article history:*
Received 2 September 2014
Accepted 20 January 2015
Available online

*Keywords:*
Heart valve
Valvular interstitial cells
Hydrogel
Elasticity
Three-dimensional cell culture
ECM

ABSTRACT

Valvular interstitial cells (VICs) are active regulators of valve homeostasis and disease, responsible for secreting and remodeling the valve tissue matrix. As a result of VIC activity, the valve modulus can substantially change during development, injury and repair, and disease progression. While two-dimensional biomaterial substrates have been used to study mechanosensing and its influence on VIC phenotype, less is known about how these cells respond to matrix modulus in a three-dimensional environment. Here, we synthesized MMP-degradable poly(ethylene glycol) (PEG) hydrogels with elastic moduli ranging from 0.24 kPa to 12 kPa and observed that cell morphology was constrained in stiffer gels. To vary gel stiffness without substantially changing cell morphology, cell-laden hydrogels were cultured in the 0.24 kPa gels for 3 days to allow VIC spreading, and then stiffened *in situ* via a second, photoinitiated thiol-ene polymerization such that the gel modulus increased from 0.24 kPa to 1.2 kPa or 13 kPa. VICs encapsulated within soft gels exhibited αSMA stress fibers (~40%), a hallmark of the myofibroblast phenotype. Interestingly, in stiffened gels, VICs became deactivated to a quiescent fibroblast phenotype, suggesting that matrix stiffness directs VIC phenotype independent of morphology, but in a manner that depends on the dimensionality of the culture platform. Collectively, these studies present a versatile method for dynamic stiffening of hydrogels and demonstrate the significant effects of matrix modulus on VIC myofibroblast properties in three-dimensional environments.



## 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 αSMA. 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 (αSMA) is a commonly used marker for myofibroblast-like cells [7,8]. The presence of organized αSMA 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.

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

* Corresponding author. Department of Chemical and Biological Engineering, University of Colorado at Boulder, Boulder, CO 80309, USA. Tel.: +1 303 492 3147; fax: +1 303 492 4341.
*E-mail address:* Kristi.Anseth@colorado.edu (K.S. Anseth).

http://dx.doi.org/10.1016/j.biomaterials.2015.01.047
0142-9612/

threshold for activation, 2D experiments generally show that lower substrate modulus (<~5 kPa) leads to mostly quiescent VICs and higher substrate modulus (>~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 αSMA 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, αSMA expression was found to increase with culture time over 14 days and was dependent on TGF-β1 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 αSMA 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-β1 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 αSMA-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 αSMA. 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% $CO_2$ 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.