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Small peptide functionalized thiol–ene hydrogels as culture substrates for understanding valvular interstitial cell activation and *de novo* tissue deposition

Sarah T. Gould [a], Nicole J. Darling [a], Kristi S. Anseth [a,b,*]

[a] Department of Chemical and Biological Engineering, University of Colorado, Boulder, CO 80303, USA
[b] Howard Hughes Medical Institute, University of Colorado, Boulder, CO, USA

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ABSTRACT

A thiol–ene polymerization platform was used to synthesize peptide functionalized poly(ethylene glycol) hydrogels, which were initially characterized and compared to theoretical predictions of Young's modulus via a theoretical crosslinking density equation presented herein. After thorough characterization, this material system's utility for answering specific biological hypotheses was demonstrated with the culture and observation of aortic valvular interstitial cells (VICs). Specifically, these materials were used to better understand the role of substrate elasticity and biochemical functionality on VIC α-smooth muscle (αSMA) expression and secretory properties (i.e. *de novo* extracellular matrix (ECM)). The Young's moduli of the hydrogels varied from 28 kPa (activating, 90% myofibroblasts) to 4 kPa (non-activating, 15% myofibroblast), and the biochemical functionality was tailored by incorporating three small adhesive peptide sequences, RGDS, VGVAPG and P15. To promote VIC adhesion, a basal [RGDS] of 0.8 mM was used in all formulations, while the [VGVAPG] or [P15] were varied to be lower than, equal to or higher than 0.8 mM. The substrates with 1.2 mM VGVAPG and all gels with P15 led to significantly higher αSMA expression for both stiff and soft substrates, as compared to 0.8 mM RGDS alone. Importantly, all gel conditions αSMA expression were significantly lower than tissue culture poly(styrene) (TCPS; ~4- to 10-fold difference). The ECM produced decreased significantly as the total integrin-binding peptide concentration increased, but was significantly higher than that produced on TCPS. This easily tailored material system provides a useful culture platform to improve the fundamental understanding of VIC biology through isolating specific biological cues and observing VIC function.

1. Introduction

Valvular interstitial cells (VICs) are the main cell population in heart valves and are responsible for maintaining valve homeostasis [1]. In healthy valves, VICs typically reside in a quiescent fibroblast state, but can be activated to form myofibroblasts upon injury to the valve [2]. In this activated state, they are known to be able to contract [1–5], proliferate [2,3,6] and express a host of different proteins, including cytokines [2,5,6], matrix metalloproteinases (MMPs) [1,2,6,7] and extracellular matrix (ECM) molecules [2,3,6–9], all of which help maintain proper valve function. However, this delicate balance of VIC activation and secretory properties can go awry if misregulation and repeated injuries occur. For example, the prolonged activation and elevated ECM expression of VICs can lead to calcific aortic stenosis [6,10–13].

The microenvironment of the valve has an acute influence on VIC phenotype and function, and researchers are only beginning to understand how valve attributes and conditions direct VIC behavior. Activation of VICs to the myofibroblast phenotype is known to depend on the culture substrate stiffness [2,6–9,14,15] and to occur in response to specific proteins presented on culture substrates [1,9,11,12,16,17]. This increase in activation due to physical or biochemical cues has also been linked to subsequent increases in ECM production [1,17,18]. Understanding the complex interplay between cellular cues and matrix interactions, and how they influence VIC secretory properties, especially the deposition of ECM, would be extremely advantageous, when trying to design bioactive culture platforms to probe and eventually direct VIC function. Todate, few studies have reported on how VIC-material interactions influence the composition and deposition of ECM produced by VICs [12,17,19]. This is further complicated by the fact that, when VICs are isolated from valve tissue and cultured on traditional tissue culture plasticware, all of the VICs become activated myofibroblasts in less than 48 h, which causes potentially non-physiologically relevant cellular responses [1].

To achieve precise control of VIC culture conditions, a thiol–ene step-growth photopolymerization was employed to fabricate a highly defined culture substrate. These materials were fabricated

* Corresponding author at: Department of Chemical and Biological Engineering, University of Colorado, Boulder, CO 80303, USA. Tel.: +1 303 492 3147.
E-mail address: Kristi.Anseth@Colorado.edu (K.S. Anseth).

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by reacting norbornene-functionalized 8-armed poly(ethylene glycol) (PEG) monomers with cysteine (i.e. thiol) functionalized small peptides in the presence of ultraviolet (UV) light and a photoinitiator. Through the simple selection of macromolecular precursors, the substrate stiffness can be manipulated by altering the molecular weight of the PEG monomers for large changes in gel modulus or performing the reaction off-stoichiometry for small modulus adjustments [20–23]. The biochemical composition of the gel is also easily tailored to the hypothesis at hand by introducing cysteine-containing peptides that are covalently bound within the gel network based on the initial reactant stoichiometry. By selecting small integrin-binding peptides derived from larger ECM molecules to incorporate into the gels, one can study the effects of the cell–matrix relationship on VIC function and remove inherent confounding factors from the system, such as cytokine sequestering [3,9,24–29]. Additionally, the use of hydrophilic PEG as the base gel formulation creates a "blank slate" culture substrate that can be functionalized with specific proteins of interest, by minimizing non-specific protein adsorption [25–27]. The small peptide adhesion sequences used herein are derived from the larger ECM molecules found in the valve tissue: fibronectin (RGDS) [30], elastin (VGVAPG) [31] and collagen-1 (P15) [32].

Here, we endeavor to design VIC culture platforms utilizing the above described PEG-based system to vary both substrate modulus and integrin-binding events to gauge the importance of these two culture conditions on the activation and ECM expression of VICs.

2. Materials and methods

2.1. Materials

Eight-armed poly(ethylene glycol) (MW: 20,000 and 40,000) was purchased from JenChem. All amino acids and resin for solid-phase peptide synthesis (SPPS) were purchased from Anaspec and Novabiochem, respectively. Porcine hearts for VIC isolation were obtained from Hormel Inc. All other chemicals were purchased from Sigma–Aldrich, unless otherwise specified.

2.2. Synthesis of eight-armed PEG-norbornene

Eight-armed PEG-norbornene (PEG-N; Fig. 1a) (MW: 20,000 and 40,000) was synthesized as previously described by Fairbanks et al. [25] Briefly, the reaction was carried out under anhydrous conditions in the organic solvent dichloromethane, where a PEG solution was added dropwise to a stirred solution of N,N′-dicyclohexylcarbodiimide and norbornene acid, and allowed to react overnight at room temperature. The norbornene-functionalized PEG in this solution was then precipitated in ice-cold ethyl ether, filtered and redissolved in chloroform. This chloroform–PEG solution was then washed with a glycine buffer and brine before being precipitated in ice-cold ethyl ether and filtered again. The filtered PEG was then placed in a vacuum chamber to remove excess ether. The percent functionalization of PEG arms with norbornene groups was determined using ^{1}H nuclear magnetic resonance imaging (NMR) and comparing the hydrogen peaks associated with the carbon adjacent to the ester linkage (~4.2 ppm) to those associated with the PEG molecule (~3.6 ppm). Supplemental Fig. 7 demonstrates typical ^{1}H-NMR spectra along with the integrated peak values. Only synthesis products with greater than 95% functionalization were used.

2.3. Synthesis of adhesive small peptide sequences and non-degradable dithiol linker peptide

Three peptides were chosen for inclusion in the thiol–ene formulation: one each from fibronectin (**C**GRGDS), elastin (**C**GVGVAPG) and collagen-1 (**C**GGTPGPQGIAGQRGVV) (i.e. CGP15). A scrambled peptide (**C**GRSGD) was also synthesized to maintain the total pendant peptide concentration at 2 mM. Although a 2 mM pendant peptide is added to the monomer solution, the final concentration available for interaction with the cells will be lower, depending on the incorporation efficiency and gel swelling. Supplemental Fig. 1 illustrates that the actual surface concentration is approximately 25% of the initial 2 mM (i.e. 500 μM), and was not significantly different between gels of the two moduli used for VIC culture (i.e. 28 and 4 kPa). To avoid confusion, results are reported with the initial monomer solution peptide concentrations used to formulate the gels, as is typical in the literature. Additionally, an MMP cleavable dicysteine (i.e. dithiol) peptide derived from collagen-1 (K**C**GGPQGIWGQG**C**K) was also selected, based on previous success in three-dimensional cultures [25,33–36]. For the purpose of the present 2-D cultures, the peptide linker was rendered non-degradable to MMPs by using the D conformation of isoleucine (I) instead of the naturally occurring L conformation (i.e. non-degradable dithiol linker peptide). These peptides were synthesized using SPPS on an Applied Biosystems model 433A or Tribute peptide synthesizer. After cleavage with 5 wt.% phenol trifluoroacetic acid and ice-cold ether precipitation, if the purity was found to be less than 95% via high performance liquid chromatography (HPLC), then large-scale HPLC purification was performed. The correct eluate fraction, based on the molecular weight, was determined by matrix-assisted laser desorption ionization. The HPLC buffer was removed from the peptide in solution via lyophilization.

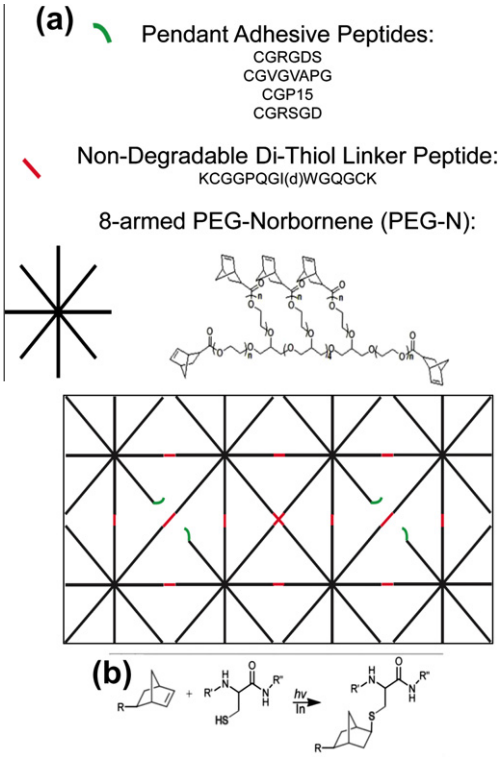

Fig. 1. (a) Monomers used for the fabrication of peptide functionalized PEG gels. The macromolecular precursors included 8-armed PEG-N (where n = 125 and 250 for 20 and 40 kDa PEG, respectively), the non-degradable dithiol linker peptide (where d denotes reversed chirality of the Ile), and the adhesive and scrambled pendant peptide sequences. These macromolecular monomers copolymerize to form a crosslinked network, as depicted with the idealized schematic of a relatively uniform mesh size. (b) Depiction of the reaction of a cysteine containing thiol with the norbornene functionality. R, R′ and R″ denote PEG, and the two adjacent amino acids in any of the peptide sequences, respectively.

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