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Research Paper

Mechanical characterization of sequentially layered photo-clickable thiol-ene hydrogels

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ABSTRACT

Multi-layer hydrogels are promising for tissue engineering due to the ability to control the local properties within each layer. However, the interface that forms between each layer has the potential to affect the performance of the hydrogel. The goals of this study were to characterize how the interface forms via its thickness and mechanical properties, identify its impact on the overall hydrogel properties, and provide new insights into how to control the interface. A photo-clickable poly(ethylene glycol) hydrogel was used to form bilayer hydrogels that were sequentially polymerized in a step-and-repeat process. Different processing conditions were studied: the time (0–20 min) before initiating polymerization of the second layer (soak time, t_s) and the hydrogel crosslink density (the same, less crosslinked, or more crosslinked) of the first layer as compared to the second layer. Interface thickness was characterized by confocal microscopy, monomer transport by Fickian diffusion, single and bilayer hydrogel mechanics by bulk moduli measurements, and interface moduli measurements using AFM, nanoindentation, and strain mapping. The interface thickness ranged from ~70 to 600 μm (1–10% of total height) depending on processing conditions, but did not affect the bulk hydrogel modulus. Analysis of monomer transport revealed that convection, due to changes in hydrogel swelling, and diffusion contribute to interface thickness. Nanomechanical analysis of bilayer hydrogels formed from soft (75 kPa) and stiff (250 kPa) layers showed a gradient in elastic modulus across the interface, which corresponded to strain maps. In summary, this work identifies that diffusive and convective transport of monomers across the interface controls its thickness and that a mechanically robust interface forms, which does not affect the hydrogel modulus. By controlling the processing conditions, the thickness of the interface can be tuned without affecting the mechanical properties of the bulk hydrogel.

1. Introduction

Many tissues in the body exist as multi-layer structures with each layer having distinct biochemical and mechanical properties that together contribute to the overall function of the tissue. Examples of such tissues include cartilage, which is composed of superficial, middle and deep zonal layers, skin, which consists of an epidermis and dermis layer, and the junction that connects cartilage to bone or tendons and ligaments to bone. Between each layered structure, an interface forms that can be characterized by either extended or abrupt gradients in the biochemical and/or mechanical properties. For example, abrupt gradients are present in the mechanical transition at the osteochondral junction between stiff calcified cartilage and compliant hyaline cartilage (Campbell et al., 2012) and in the compositional transition across the muscle-tendon junction (Ker, 2007). The ability to mimic these complex tissue structures in a scaffold is an important step towards developing successful strategies for tissue engineering composite tissues.

Multi-layered hydrogels can be fabricated such that their chemistry and properties (e.g., crosslinking and porosity) vary in space. Mild polymerization conditions coupled with a high water content make hydrogels particularly suitable for the encapsulation and culture of cells in 3D scaffolds (Nicodemus and Bryant, 2008).

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As such, multi-layer hydrogels are being designed and investigated for tissue engineering of complex multi-structured tissues. For example, multi-layer fibrous hydrogels made from chitosan were designed where keratinocytes were seeded into a layer simulating the dermis, fibroblasts seeded into a second layer representing the protective epidermis, and a thin film to mimic the basement membrane to engineer a skin substitute (Lin et al., 2015). This tri-layered hydrogel enabled both cell types to replicate the striation of full thickness skin more accurately and in a shorter time than single or bilayer counterparts. In a separate study, a multi-layer hydrogel formed from crosslinked poly(ethylene glycol) (PEG) was tuned by introducing different extracellular matrix molecules into each layer to mimic the chemical makeup of the different zones in cartilage (Nguyen et al., 2011). In doing so, 3D hydrogel niches were created that allowed a single population of stem cells to differentiate into zone-specific chondrocytes. In another study, the composition and mechanical properties in each layer of a cross-linked PEG hydrogel was varied to capture the physiochemical cues that arise in osteochondral tissues under mechanical loads (Steinmetz et al., 2015). When human mesenchymal stem cells were encapsulated in the multi-layer hydrogel and subjected to dynamic loading, differentiation towards a chondrogenic phenotype was observed in one layer and an osteogenic phenotype was observed in the other layer. Taken together, these examples among others demonstrate that multi-layered hydrogels are promising for tissue engineering complex multi-layer composite tissues.

In forming multi-layer hydrogels, the interface that forms between two adjacent layers is critical to the overall function of the hydrogel. For example, if the layers are not well integrated and secured together, the interface is prone to shear failure and can lead to delamination (Kandel et al., 2006; Sherwood et al., 2002). For interfaces with abrupt gradients, the two dissimilar materials disrupt strain transfer and can lead to areas of high stress concentration that result in failure at the interface (Erdogan, 1995). On the contrary, if the interface that forms is large, the local biochemical and mechanical properties can influence the fate of cells embedded within this region (Steinmetz et al., 2015). Thus, controlling the interface is important to designing multi-layer hydrogels that recapitulate extended or abrupt gradients found in native interfaces and that lead to mechanically robust interfaces to minimize failure. The resulting interface when multi-layer hydrogels are formed has received little attention.

This study investigates a bilayer PEG hydrogel and the interface that forms between the two layers. PEG hydrogels were chosen for their promise in producing multi-layer hydrogels for use in vascular (Fischer et al., 2015; Shinohara et al., 2013) and musculoskeletal (Fuoco et al., 2012; Hwang et al., 2010; Lin et al., 2014; Nguyen et al., 2012; Paxton et al., 2009; Steinmetz et al., 2015) tissue engineering. The objectives for this study are to characterize how the interface forms via its thickness and mechanical properties, identify its impact on the overall hydrogel properties, and provide new insights into how to control the interface. Bilayer PEG hydrogels were sequentially polymerized from photo-clickable thiol-ene macromolecular monomers. This reaction scheme was chosen for its promise in tissue engineering (Fairbanks et al., 2009; Tibbitt and Anseth, 2009) and its highly specific, efficient, and rapid reaction (Hoyle and Bowman, 2010). Specifically, this study investigated (a) the effects of monomer transport between the two layers as a function of processing conditions on interface thickness and bulk mechanical properties and (b) the local mechanical properties and strain transfer across the interface when each hydrogel layer is formed with different mechanical properties. The latter is important for applications where mechanical forces are applied to multi-layer hydrogels. Overall, this study provides new insight into how processing parameters influence the formation and properties of the interface and ultimately the contribution that the interface has on the macroscopic properties. Findings from this study will aid the development of multi-layer hydrogels where the interface can be tuned to create thin interfaces that lead to abrupt property changes across the interface or thick interfaces where large property gradients across the interface are required.

2. Materials and methods

2.1. Monomer synthesis

8-arm PEG with terminal amines (20,000 g/mol; JenKem Technology USA, Plano, TX) was functionalized with norbornenes by reacting 5-norbornene-2-carboxylic acid (Sigma-Aldrich, St. Louis, MO) with 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uranium hexafluorophosphate methanaminium (HATU) (Chem-Impex International, Inc., Wool Dale, IL), and N,N-diisopropylethylamine (DIPEA) (Chem-Impex) in dimethylformamide (DMF)/dichloromethane (DCM) (Sigma-Aldrich). The reaction was allowed to proceed overnight at room temperature under an inert atmosphere. The product was precipitated in diethyl ether (Sigma-Aldrich), filtered, dialyzed, and lyophilized. The extent of conjugation of norbornene to each arm of the 8-arm PEG-amine was determined to be 92% using ^{1}H NMR by comparing the protons across the carbon-carbon double bond in the norbornene to the methylene protons in PEG.

2.2. Fabrication of PEG hydrogels

A precursor solution consisting of 8-arm PEG-norbornene (PEGnor) monomer, PEG-dithiol (PEGdt) crosslinker (1000 or 3400 g/mol; Sigma-Aldrich) at 1:1 thiol:ene ratio (assuming 100% norbornene conjugation), and 0.05% (w/w) photoinitiator, 1-(4-(2-Hydroxyethoxy)-phenyl)-2-hydroxy-2-methyl-1-propane-1-one (I2959; Ciba Specialty Chemicals, Tarrytown, NY), in deionized water (diH_2O) was photopolymerized by ultraviolet light (10 min, 5–10 mW/cm^2, 352 nm). The concentration of 8-arm PEG-norbornene varied by 10, 15 or 25% (w/w) (Hydrogel I, II/III and IV, respectively) depending on the experiment. Hydrogels were formed in either a cylindrical or rectangular geometry depending on the experiment (as described below). For single layer constructs, full height (i.e., 5 mm) hydrogels were fabricated from a single macromer solution. For bilayer constructs, half-height (i.e., 2.5 mm) hydrogels were fabricated from one macromer solution that was polymerized to form the first layer. Previous studies have reported that complete polymerization of similar precursor formulations is reached in less than a minute (Roberts and Bryant, 2013) and therefore ten minutes ensures that the first layer has polymerized completely. Following polymerization of the first layer, a second macromer solution was carefully deposited on top of the first layer to form the second layer and to reach full height (i.e., 5 mm). The second solution was left to allow for transport of the monomers into the first hydrogel layer for prescribed periods of time (t_s), after which the second layer was polymerized. A schematic representation of the fabrication process is shown in Fig. 1A.

2.3. Swelling studies and hydrogel characterization

Single layer, full height cylindrical hydrogels prepared as stated above were weighed immediately after polymerization and then allowed to swell in diH_2O for 48 h at room temperature. Equilibrium swollen hydrogels ($n=3$/group) were weighed to determine the equilibrium swollen mass. The dry polymer mass was obtained for each hydrogel after lyophilization. The equilibrium mass swelling ratio, q, was calculated by dividing the equilibrium swollen mass by the polymer dry mass. The equilibrium swelling