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Enzyme-mediated stiffening hydrogels for probing activation of pancreatic stellate cells

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ABSTRACT

The complex network of biochemical and biophysical cues in the pancreatic desmoplasia not only presents challenges to the fundamental understanding of tumor progression, but also hinders the development of therapeutic strategies against pancreatic cancer. Residing in the desmoplasia, pancreatic stellate cells (PSCs) are the major stromal cells affecting the growth and metastasis of pancreatic cancer cells by means of paracrine effects and extracellular matrix protein deposition. PSCs remain in a quiescent/dormant state until they are 'activated' by various environmental cues. While the mechanisms of PSC activation are increasingly being described in literature, the influence of matrix stiffness on PSC activation is largely unexplored. To test the hypothesis that matrix stiffness affects myofibroblastic activation of PSCs, we have prepared cell-laden hydrogels capable of being dynamically stiffened through an enzymatic reaction. The stiffening of the microenvironment was created by using a peptide linker with additional tyrosine residues, which were susceptible to tyrosinase-mediated crosslinking. Tyrosinase catalyzes the oxidation of tyrosine into dihydroxyphenylalanine (DOPA), DOPA quinone, and finally into DOPA dimer. The formation of DOPA dimer led to additional crosslinks and thus stiffening the cell-laden hydrogel. In addition to systematically studying the various parameters relevant to the enzymatic reaction and hydrogel stiffening, we also designed experiments to probe the influence of dynamic matrix stiffening on cell fate. Protease-sensitive peptides were used to crosslink hydrogels, whereas integrin-binding ligands (e.g., RGD motif) were immobilized in the network to afford cell-matrix interaction. PSC-laden hydrogels were placed in media containing tyrosinase for 6 h to achieve *in situ* gel stiffening. We found that PSCs encapsulated and cultured in a stiffened matrix expressed higher levels of αSMA and hypoxia-inducible factor 1α (HIF-1α), suggestive of a myofibroblastic phenotype. This hydrogel platform offers a facile means of *in situ* stiffening of cell-laden matrices and should be valuable for probing cell fate process dictated by dynamic matrix stiffness.

Statement of Significance

Hydrogels with spatial-temporal controls over crosslinking kinetics (i.e., dynamic hydrogel) are increasingly being developed for studying mechanobiology in 3D. The general principle of designing dynamic hydrogel is to perform cell encapsulation within a hydrogel network that allows for postgelation modification in gel crosslinking density. The enzyme-mediated *in situ* gel stiffening is innovative because of the specificity and efficiency of enzymatic reaction. Although tyrosinase has been used for hydrogel crosslinking and *in situ* cell encapsulation, to the best of our knowledge tyrosinase-mediated DOPA formation has not been explored for *in situ* stiffening of cell-laden hydrogels. Furthermore, the current work provides a gradual matrix stiffening strategy that may more closely mimic the process of tumor development.

1. Introduction

The stiffness of extracellular matrices (ECM) increases during the progression of many diseases, including cancers [1,2]. The

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implications of a stiffened tissue include abnormal intracellular mechano-sensing and signal transduction, increased expression of ECM proteins, and durotaxis of cells (i.e., cell migration guided by gradients of matrix rigidity). Seminal works concerning the influence of matrix mechanics on cell fate processes relied largely on two-dimensional (2D) polyacrylamide hydrogels with surface-adsorbed or conjugated ECM proteins [3]. Culturing cells on a 2D surface, however, does not truly recapitulate the complex architecture of three-dimensional (3D) ECM [4]. In this regard, cell-laden hydrogels are increasingly being explored for modeling disease progression *in vitro* or *ex vivo*, as well as for regenerating tissues *in vivo*. The high water content and good permeability of a highly swollen hydrogel permits facile nutrient-waste exchange, whereas the crosslinked polymeric network gives rise to tunable elasticity and easy tethering of bioactive motifs for supporting cell survival and function in 3D [5]. Hydrogels prepared from synthetic polymers, such as poly(ethylene glycol) (PEG), are particularly suitable for investigating the influence of extracellular matrix mechanics on cell fate because the crosslinking density, and hence the stiffness, of a biomimetic hydrogel can be precisely engineered [5].

In recent years, hydrogels with dynamically tunable crosslinking kinetics are increasingly being explored for studying mechanobiology in 3D [6–8]. The general principle of designing a dynamic hydrogel is to perform cell encapsulation within a primary hydrogel network that allows for post-gelation modification in gel crosslinking density. Employing a two-stage crosslinking strategy, Burdick and colleagues showed that cell-laden hydrogels could be stiffened in a spatial-temporally controlled manner [8]. The hydrogels were first prepared by thiol-based Michael-type addition between methacrylated hyaluronan (MeHA) and dithiothreitol (DTT) with an off-stoichiometric ratio. The excess methacrylate moieties in the primary hydrogel network permit subsequent light-and-radical-mediated chain-growth polymerization to produce a stiffened gel matrix. Anseth et al. showed that a stiffened hydrogel matrix could be achieved simply by performing a secondary step-growth photopolymerization in the presence of a pre-gelled cell-laden hydrogel network [9]. *In situ* hydrogel stiffening could also be achieved through light irradiation. For example, PEG-based hydrogels with azobenzene were prepared to undergo reversible swelling upon azobenzene *cis-trans* isomerization, which is induced by UV or visible light exposure, respectively [10]. Although the azobenzene-linker chain length, and hence hydrogel swelling, could be modulated by light exposure, the magnitude of gel modulus change was minimal and not physiologically relevant. Alternatively, infrared (IR)-induced heating was used to tune the stiffness of alginate hydrogels across a physiologically relevant range [11]. In this example, temperature-sensitive liposomes were loaded with gold nanorods as well as calcium, and were subsequently encapsulated in the alginate gels. Upon IR irradiation, the heated gold nanorods disrupt the liposomes, causing the release of calcium ions to induce gelation of alginate chains. Although IR light is considered safer than UV light, the generation of heat upon IR irradiation might not be ideal for certain applications. Our group has utilized host-guest (cyclodextrin-adamantane) interactions to reversibly tune the stiffness of cell-laden hydrogels across several hundreds to thousands of Pascals [12]. Collectively, these approaches provide a wide variety of options for irreversibly or reversibly tuning the stiffness of cell-laden hydrogels.

Owing to their substrate specificity and predictable enzymatic reaction kinetics, various enzymes (e.g., plasmin, transglutaminase, horseradish peroxidase, glucose oxidase, and tyrosinase) have been successfully used to induce gel crosslinking and, in some cases, cell encapsulation [13–17]. For example, tyrosinase (also named polyphenol oxidase) catalyzes the oxidation of phenol into dihydroxyphenylalanine (DOPA), DOPA quinone, and subsequently into DOPA dimer [18]. Tyrosinase-mediated reactions also consume molecular oxygen and produce water as the only by-product. Tyrosine or DOPA conjugated polymers (e.g., PVA, gelatin, dextran, etc.) are susceptible to tyrosinase-mediated crosslinking [19]. Due to its mild reaction conditions, tyrosinase is increasingly being explored for hydrogel crosslinking and *in situ* cell encapsulation [14]. To the best of our knowledge, however, tyrosinase-mediated DOPA crosslinking mechanism has not been exploited for *in situ* stiffening of cell-laden hydrogels. While tyrosinase-mediated DOPA formation was not found in the pancreatic tissue, this strategy provides facile, effective, and cytocompatible means of tuning matrix stiffness for *in vitro* or *ex vivo* tissue engineering applications.

In this contribution, we describe the design of orthogonally crosslinked PEG-peptide thiol-norbornene hydrogels susceptible to tyrosinase-mediated *in situ* gel stiffening. The primary hydrogel network was prepared by a light-mediated thiol-norbornene photopolymerization [20,21] utilizing bis-cysteine-bis-tyrosine-bearing peptide crosslinkers. The pendant tyrosine residues in the primary step-growth hydrogel network permit additional crosslinking and gel stiffening triggered by the infiltration of tyrosinase. In addition to verifying the formation of DOPA crosslinks within a PEG-peptide hydrogel network, we also optimized the conditions for achieving a biologically relevant range of stiffening. Finally, we utilized this *in situ* stiffening PEG-peptide hydrogel system to probe the effect of a stiffened matrix on the activation of pancreatic stellate cells.

2. Materials & methods

2.1. Materials

Hydroxyl-terminated 8-arm PEG (20 kDa) and 5-norbornene-2-carboxylic acid was obtained from JenKem Technology USA and Sigma-Aldrich, respectively. All reagents for chemical synthesis were purchased from Sigma-Aldrich unless otherwise noted. Reagents and Fmoc-amino acids for solid phase peptide synthesis were acquired from Anaspec or ChemPep.

2.2. Modeling of tyrosinase diffusion in hydrogels

The time-scale of tyrosinase diffusion into PEG-peptide hydrogel was estimated by Fick's 2nd Law of Diffusion in planar geometry (Eq. (1)) with appropriate initial (Eq. (2)) and boundary conditions (Eqs. (3) and (4)) as listed below:

$$\frac{\partial C_T}{\partial t} = D_T \frac{\partial^2 C_T}{\partial z^2} \tag{1}$$

$$@\ t = 0\ C = 0 \tag{2}$$

$$@\ z = 0\ \frac{\partial C_T}{\partial z} = 0 \tag{3}$$

$$@\ z = \pm h\ C_T = C_0 \tag{4}$$

Here, C_T and D_T are the concentration and diffusion coefficient of tyrosinase in a hydrogel, respectively; z is the coordinate perpendicular to the gel; t is the time of gel incubation in tyrosinase solution; h is the half-thickness of the gel; C_0 is the tyrosinase concentration in the buffer solution, which is assumed to be constant. Diffusion in this case was assumed to be symmetrical and only in the z-direction since the gels' diameter (~10 mm) was much greater than its thickness (<1 mm). Eqs. (1)–(4) were solved using Polymath software using a range of diffusion coefficients ($\sim 9 \times 10^{-8} - 5 \times 10^{-7}$ cm^2/s) and gel thickness (~0.5 – 1 mm). The time needed for C_T at the center plane of the gel (i.e., $z = 0$) to reach 99% of that in solution