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Evaluation of photochemistry reaction kinetics to pattern bioactive proteins on hydrogels for biological applications

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ABSTRACT

Bioactive signals play many important roles on cell function and behavior. In most biological studies, soluble biochemical cues such as growth factors or cytokines are added directly into the media to maintain and/or manipulate cell activities *in vitro*. However, these methods cannot accurately mimic certain *in vivo* biological signaling motifs, which are often immobilized to extracellular matrix and also display spatial gradients that are critical for tissue morphology. Besides biochemical cues, biophysical properties such as substrate stiffness can influence cell behavior but is not easy to manipulate under conventional cell culturing practices. Recent development in photocrosslinkable hydrogels provides new tools that allow precise control of spatial biochemical and biophysical cues for biological applications, but doing so requires a comprehensive study on various hydrogel photochemistry kinetics to allow thorough photocrosslink reaction while maintain protein bioactivities at the same time. In this paper, we studied several photochemistry reactions and evaluate key photochemical parameters, such as photoinitiators and ultra-violet (UV) exposure times, to understand their unique contributions to undesired protein damage and cell death. Our data illustrates the retention of protein function and minimize of cell health during photoreactions requires careful selection of photoinitiator type and concentration, and UV exposure times. We also developed a robust method based on thiol-norbornene chemistry for independent control of hydrogel stiffness and spatial bioactive patterns. Overall, we highlight a class of bioactive hydrogels to stiffness control and site specific immobilized bioactive proteins/peptides for the study of cellular behavior such as cellular attraction, repulsion and stem cell fate.
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1. Introduction

The development of bioactive materials is vital for introducing new methods to study and manipulate cell behavior. Currently, the majority of *in vitro* cell studies rely on tissue culture plastic (TCP) as a culture substrate and soluble biochemical cues for regulating cell activities. These methods, however, are unable to accurately display certain signaling motifs found *in vivo* environments, including immobilized growth factors, cell-cell ligand-receptor interactions, and spatially localized signaling. Furthermore, biophysical cues such as tissue stiffness have important roles in cell phenotype [1–3], whereas TCP does not provide physiologically relevant stiffness. To overcome these challenges, bioactive materials are excellent candidates for mimicking tissue stiffness, immobilizing biomolecules, and creating spatially specific biochemical patterns. Specifically tailored hydrogels have been previously successful in influencing cell morphology [4], cell function [5,6] as well as stem cell fate [3].

Abbreviations: UV, ultra-violet; ECM, extracellular matrix; TCP, tissue culture plastic; PEG, polyethylene glycol; PEGDA, polyethylene glycol diacrylate; DMPA, 2-dimethoxy-2-phenylacetophenone; LAP, lithium phenyl-2,4,6-trimethylbenzoylphosphinate; VEGF, vascular endothelial growth factor; EC, endothelial cell; mESC, mouse embryonic stem cell; HUVEC, human umbilical vein endothelial cell.

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Hydrogels are commonly used as cell scaffolds due to their biophysical and biochemical commonalities with the extracellular matrix (ECM) [7,8]. Natural polymers, such as collagen or fibrinogen, are common choices for scaffolds as they are biocompatible and found in many tissues of the body, but they lack the easily targeted chemical moieties for bioconjugation purposes. Synthetic hydrogels are advantageous for the manipulation of both stiffness and bioactive molecule attachment. In particular, polyethylene glycol (PEG) is a biologically inert synthetic polymer commonly utilized as a blank slate for cell scaffolds [9,10]. Photopolymerization can be used for PEG hydrogel formation and bioconjugation [10]. Photochemistry requires a photoinitiator and ultra-violet (UV) light exposure to initiate and propagate the reaction. When photoinitiators are introduced to UV light, chemical bonds break to form radicals. Theses radicals are critical for the reaction initiation but can also negatively affect proteins or cells that are present [11]. Two common photoinitiators used in biorelated PEG crosslinking include 2-dimethoxy-2-phenylacetophenone (DMPA) and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) [12,13]. Specifically, DMPA has been often used in combination with acrylate PEG chemistry [10,12]. LAP has recently reported in studies utilizing thiol-ene reactions [13].

Additionally, there are different photo-reactive chemistries employed for PEG hydrogel synthesis. Acrylate hydrogels entail PEG-diacrylate (PEGDA) monomers undergoing acrylate-acrylate chain growth polymerization resulting in randomly crosslinked networks [12,13]. Another approach is thiol-ene based chemistry [14,15]. Differently, thiol-ene reactions create uniform crosslinks via step growth polymerization and therefore require two reactants: PEG-dithiol monomers and multi-arm PEG containing an “ene” carbon double bond functional group. Thiol-ene, also known as click reactions, can be employed with a variety of “ene” functional groups. Moreover, these functional groups exhibit different reaction kinetics [16] and therefore offer several choices depend on the specific requirement of the applications.

In this study, we investigated how photoinitiator type and concentration influences these photochemistry kinetics, retention of protein bioactivity, and cell viability across a range of UV exposure times. Additionally, we explore both acrylate and thiol-ene PEG hydrogel substrates for stiffness manipulation and surface protein patterning. Finally, we created bioactive hydrogels with vascular endothelial growth factor (VEGF) and ephrinB2 demonstrating its impact on endothelial cell (EC) behavior.

2. Material and methods

2.1. Photoinitiator stock solutions

DMPA (Sigma Aldrich) was dissolved at a concentration of 300 mg/mL in N-Vinylpyrrolidone (NVP, Sigma Aldrich). The LAP was synthesized according to [13]. LAP stock solution was made at a 25 mg/mL in phosphate buffered saline solution. Solutions were passed through 0.22 μm filer for sterilization. Stock solutions were protected from the light with aluminum foil and stored at 4 °C.

2.2. Thiol-ene reaction kinetics

A free thiol colorimetric detection assay, Ellman's Reagent (Thermo Sci.), was used to measure thiol-ene reaction kinetics. In short, the peptide, Arginine-Glycine-Aspartic acid (RGD), was purchased with an additional cysteine residue at the terminus to provide a free thiol for bioconjugation (RGDC, American Peptide). Reduced peptide aliquots were stored in −20 °C to maintain free thiols over time (Supplemental Fig. S1). RGDC was allowed to react with an 8-arm PEG norbornene (JenKem Tech, 20 KDa) at a one to eight molar ratio of norbornene to thiol. The reaction was measured with Ellman's assay after various UV light wavelengths (365 nm or 390 nm) and exposure times, ranging from 10 to 60 s. The amount of free thiols present was calculated and analyzed as inversely proportional to the percentage of reaction that has occurred. Thiol-ene kinetics are reported as a time course and free thiols present are compared to the amount of thiols measured prior to the reaction starts.

2.3. Lysozyme bioactivity assay

To evaluate the bioactivity of proteins after photoreactions, an assay was developed using lysozyme as a model protein. In short, lysozyme (Sigma Aldrich) was exposed to different photoinitiator concentrations and UV light times. For a negative control, lysozyme was boiled for 10 min at 90 °C. Untreated lysozyme served as a positive control. Next, treated lysozyme solutions (1 mg/ml) were added to an overnight *E. coli* culture and incubated for 4 h at room temperature. Solutions were centrifuged and the supernatant was collected for analysis. Bradford assay (Bio-Rad) was used to measure bacteria lysate collected from each treatment group. As expected, the supernatant collected from incubated boiled lysozyme measured no additional protein in the solution; on the other hand, untreated lysozyme measured high concentrations of proteins from the successfully lysed bacteria. Bioactivity is reported as a percentage calculated from the positive control of the assay.

2.4. Cell culture and hydrogel seeding

Mouse embryonic stem cells (mESC) were cultured in 37 °C in 5% CO_2 maintained on 0.1% gelatin coated dishes with ESGRO complete defined serum-free medium (Stem Cell Technologies) with a selective GSK3beta inhibiter. Media was replaced every two days and routinely passaged. For hydrogel seeding, mESCs were added at a cell density of 200,000 cells/cm^2 to various stiffness acrylate hydrogels. Human umbilical cord venous endothelial cells (HUVECs, Lonza) were cultured in 37 °C in 5% CO_2 on 0.1% gelatin coated dishes with Endothelial Cell Growth Medium-2 (EGM-2, Lonza). Media is replaced every two days and passaged regularly. HUVECs were seeded onto patterned hydrogels at a cell density of 70,000 cells/cm^2. HUVECs seeded onto VEGF immobilized hydrogels were cultured with EGM-2 without growth factors.

2.5. Cell viability measurement

To investigate the health of cells that were present during a photoreaction, a photoinitiator was introduced to a cell suspension of HUVECs and exposed to UV light for a specific amount of time. After treatment, HUVECs were seeded into a 96 well plate. After 4 h, cell viability was evaluated using live (calcein AM) dead (ethidium homodimer-1) staining following kit instructions (Thermo Fisher Scientific). As a positive control, HUVECs with no photoinitiator or UV exposure were also seeded and imaged. Additionally, solvent NVP, used in DMPA but not LAP stock solution, was tested to determine its contribution to cell viability (Supplemental Fig. S2). Images were analyzed with ImageJ software to calculate percent cell viability.

2.6. PEG-diacrylate and thiol-ene hydrogel synthesis

Acrylate-acrylate hydrogels were synthesized using polyethylene glycol diacrylate monomers (PEGDA, Laysan Bio, 3.4 kDa). Thiol-ene hydrogels were synthesized by a reaction between PEG-dithiol (Laysan Bio, 3.4 kDa) and 8-arm PEG norbornene (JenKem

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