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Photo-tunable hydrogel mechanical heterogeneity informed by predictive transport kinetics model

A photo-tunable hydrogel with elastic modulus and crosslinking was characterized *via* atomic force microscopy and confocal fluorescence microscopy, respectively, with a zoom-in on the modelled molecular dynamics during the patterning process.



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Introduction

Local control of the mechanical and chemical properties of the extracellular matrix surrounding cells is a growing need in regenerative and personalized medicine.^{1–3} Stem cell differentiation and proliferation is significantly affected by the extracellular environment, which is a cornerstone to tissue engineering.^{4,5} For example, Engler *et al.* demonstrated that the stiffness of the hydrogel to which mesenchymal stem cells (MSCs) attach controlled differentiation into bone precursor cells, muscle precursor cells, or neural precursor cells.⁵ The ability to locally define the mechanical properties that cells sense has the potential to spatially control cellular differentiation and proliferation.^{2,3,6,7}

Three-dimensional (3D) printing is a promising technique to fabricate structures that emulate the heterogeneous environments of biological tissues. Nozzle-based or 'bio-plotting' systems are a common printing method to create desired structures due to the flexibility of multiple materials and easily sterilizable

Photo-tunable hydrogel mechanical heterogeneity informed by predictive transport kinetics model[†]

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Understanding the three-dimensional (3D) mechanical and chemical properties of distinctly different, adjacent biological tissues is crucial to mimicking their complex properties with materials. 3D printing is a technique often employed to spatially control the distribution of the biomaterials, such as hydrogels, of interest, but it is difficult to print both mechanically robust (high modulus and toughness) and biocompatible (low modulus) hydrogels in a single structure. Moreover, due to the fast diffusion of mobile species during printing and nonequilibrium swelling conditions of low-solids-content hydrogels, it is challenging to form the high-fidelity structures required to mimic tissues. Here a predictive transport and swelling model is presented to model these effects and then is used to compensate for these effects during printing. This model is validated experimentally by photopatterning spatially distinct hydrogel elastic moduli using a single photo-tunable poly(ethylene glycol) (PEG) pre-polymer solution by sequentially patterning and in-diffusing fresh pre-polymer for further polymerization.

printing environments. However, the material and resolution constraints of bio-plotting severely inhibit its ability to recapitulate the microenvironment at cellular lengthscales.⁸⁻¹⁰

Stereolithography (SLA) or digital light processing (DLP), which utilizes a photoreactive liquid to iteratively build 3D structures layer-by-layer using patterned light, does not have the fundamental material restrictions of bio-plotting. As a result, applications using SLA are gaining interest due to greater control in achieving chemical and mechanical properties in 3D.^{1,11,12} However, there are several shortcomings. Traditional SLA resins, which target high fidelity printed features, are near 100% monomer concentration and have high viscosities (1 Pass) to minimize diffusion of species during photopatterning. Printing cytocompatible precursor materials is inherently different because cells require an aqueous environment to survive.¹³ Solutions of hydrogel precursor are difficult to photopattern at high resolution because their low solids content and high diffusivity of the monomers, which reduce reaction rates and increase transport rates during polymerization. Moreover, printed hydrogels create a concentration gradient in monomer that causes swelling with the resin solution, distorting the solid structure during printing. These factors reduce the fidelity of photopatterned features in cytocompatible materials. These challenges were shown in Linnenberger et al.,¹⁴ which used poly(ethylene glycol) dimethacrylate (PEGDMA) precursors and reported that to obtain stereolithography of $\sim 10 \ \mu m$ features, the precursor solution had to be polymerized to just below gelation in order to increase oligomer molecular weight and solution viscosity.

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Transport of monomers and oligomers across photopattern boundaries locally modifies the prescribed resolution, severely complicating the multiple patterning steps required for 3D SLA.

An additional consideration in DLP systems is photoinitiator concentration, which is typically chosen to avoid its significant depletion (*i.e.* concentration of photoinitiator remains effectively constant) and the associated variation in photosensitivity during the photopatterning. For this reason, significant gradients in conversion due to diffusion of photoinitiator are not expected. Conversely, the study of oxygen dynamics in these printers find that O2 is rapidly eliminated from the resin except in a small thickness near the PDMS.^{15–18} Thus, while a complete model would benefit from tracking all resin components in 3D, this work focuses on the novel aspects of radical and monomer transport, which demonstrably dominate feature development.¹⁹

Presented here is a model to predict how printing fidelity is affected by precursor solution reactivity, diffusivity, and swelling during polymerization. Specifically, we describe the characteristic diffusion distance of reactive resin species and the hydrogel matrix swelling behavior. The photopolymerization reaction kinetics model developed by Reddy *et al.*²⁰ for the cytocompatible and photo-click chemistry of thiol–norbornene are first discussed to obtain the relationship between reaction rate and specie concentration.

Diffusion and simultaneous polymerization are then shown to obey first-order steady state kinetics, from which the characteristic transport distances was derived for each species into and out of the photopatterned region (Fig. 1a). As swelling in photopatterned regions may influence patterned structure fidelity, differential swelling after polymerization is also derived from bulk equilibrium swelling properties for the photopatterned hydrogel material (Fig. 1b).

The results from the model were then experimentally validated in thiol–norbornene hydrogels which were uniformly photopolymerized, then swollen with fresh precursor solution, and finally photopatterned. As demonstrated by Fiedler *et al.*,²¹ cross-link density and modulus were increased in multiply-exposed regions by using a precursor solution that has a functional group stoichiometric ratio of 0.5:1 (thiol:ene). The initial hydrogel thus includes unreacted norbornene groups to which



Fig. 1 Illustration depicting (a) diffusion of monomer [M] into and radicals [M]] out of the photopattern and (b) differential swelling occurring due to enhanced crosslinking inside of the photopattern resulting in the movement of the polymer network [P] away from the pattern edge for the two-stage polymerization presented.

fresh thiol will react when subsequently photopatterned. Confocal fluorescence microscopy (CFM) was used to map reacted thiol concentration and predict local modulus from bulk characterization. This spatially-variable modulus was compared to that obtained by atomic force microscopy (AFM).

Experimental procedures‡

Materials

Poly(ethylene glycol) dithiol (PEG-dithiol) (Sigma Aldrich, M_W 1 kg mol⁻¹), poly(ethylene glycol) thiol (tripentaerythritol) (JenKem USA, 8ARM-PEG-SH, M_W 10 kg mol⁻¹), lithium phenyl-2,4,6trimethylbenzoylphosphinate (LAP) (Colorado Photopolymer Solutions), and fluorescent molecule AlexaFluor-546 maleimide (ThermoFisher) were used as received. 5-Norbornene-2-carboxylic acid (NB) (Sigma Aldrich) was conjugated to poly(ethylene glycol)amine (PEG-NH2) (JenKem USA, 8 arm PEG amine, HCl salt, $M_{\rm W}$ 10 kg mol⁻¹) at room temperature (RT) under an argon purge to produce poly(ethylene glycol) norbornene (PEG-NB).¹³ This was done by dissolving PEG-NH2 (10 g) in dimethylformamide (DMF) (15 mL) and dichloromethane at 1:1 ratio to which the solution containing 4-molar excess NB (4.42 g), 2 molar excess 2-(1H-7azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uranium hexafluorophosphate methanaminium (HATU, AKSci) (9.12 g), and 2 molar excess N,N-diisopropylethylamine (DIEA, Sigma) (6.2 g) was reacted for 48 hours. The solution was precipitated in diethyl ether, dialyzed four times with deionized (DI) water over two days, and lyophilized. The resulting 8-arm PEG-NB product had 99% conjugation (percentage of NB conjugated PEG arms), referred to herein as PEG-NB. The degree of norbornene conjugation was determined by proton nuclear magnetic resonance (¹H NMR, Bruker AV-III 400) by comparing the area under the peak for the allylic hydrogen closest to the norbornene bridged cyclic hydrocarbon group (resonance from $\cong 3.1$ ppm to $\cong 3.2$ ppm) to the area under the peak for the methyl groups in the PEG backbone (resonance from \approx 3.4 ppm to 3.85 ppm), see ESI,[†] Section S1 and Fig. S1 for ¹H NMR spectrum. Macromers were dissolved in phosphate-buffered saline (PBS) (OmniPur, Calbiochem).

Photopatterned sample preparation

Poly(ethylene glycol) (PEG) hydrogels formed from the thiolnorbornene photo-click polymerization was chosen for three reasons. (1) Step-growth polymerizations offer enhanced control of property variations with respect to chain-grown reactions because precise off-stoichiometry can be engineered and an excess number of functional groups can be accurately predicted. (2) The photo-click reaction between PEG-dithiol ([SH]) and PEGnorbornene ([C==C]) is cytocompatible, which is a requirement for printing in the presence of cells.^{22–24} (3) The thiol–norbornene

[‡] Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

reaction has the highest reaction rate k of known thiol-ene reactions ($k \cong 10^6 \text{ mol } \text{L}^{-1} \text{ s}^{-1}$), significantly greater than methacrylates ($k \cong 10^2 \text{ mol } \text{L}^{-1} \text{ s}^{-1}$) and acrylates ($k \cong 10^3 \text{ mol } \text{L}^{-1} \text{ s}^{-1}$).²⁰ Additionally, the lithium phosphinate (LAP) photoinitiator was chosen for its solubility in water, for its cytocompatibility and because it initiates at visible wavelengths (*e.g.*, 400 nm), which are more cytocompatible than ultraviolet wavelengths (<365 nm).²⁵

The thiol–norbornene precursor solution was selected with an off-stoichiometric ratio of 0.5:1 ([SH]:[C=C]) to ensure that 50% of the initial concentration of norbornene groups remain unreacted after initial polymerization. Once polymerized, the hydrogel was swollen with fresh precursor solution to equilibrium. At this point, the effective concentration of thiol to norbornenes present decreased from 0.5:1 to 0.33:1 due to the presence of excess [C=C] attached to the original hydrogel network.²¹

The NB solution was prepared from 8.55% (g g⁻¹) 10 kg mol⁻¹ PEG-NB in phosphate buffered saline and stored overnight at 3 °C. Immediately prior to photopolymerization, 1.45% (g g⁻¹) 1 kg mol⁻¹ PEG-dithiol was added to the NB solution with 0.05% (g g⁻¹) LAP to yield a 10% (g g⁻¹) monomer concentration. The photopatterning precursor solution was synthesized using the same method with the addition of a thiol fluorescent tag. AlexaFluor 546 maleimide which was reacted with PEG-dithiol overnight at 3 °C at a ratio of 1:3000 (maleimide:thiol) to minimize fluorophore contributions to the final material properties. As shown in Fig. 2, the precursor solution was deposited between a methacrylate coated glass slide (Cell Associates) and a RainX coated coverslip with 12.5 µm shims (optically thin for this SLA system with a depth of focus of 43 µm) and secured using clips.

The methacrylate-coated substrate was used to promote covalent bonding of the fabricated hydrogel to the glass, while the RainX was used to minimize adhesion between the fabricated hydrogel and the coverslip window. The sample was flood-exposed for 120 s using a collimated mercury lamp source



Fig. 2 Schematic of the sample preparation, in-swelling with the fluorescently-tagged thiol precursor solution, and then photopatterning using the SLA system in ESI, \dagger Fig. S2.

at 30 mW cm⁻² to ensure uniform, full conversion across and through the sample. The sample was then swollen for ~3600 s with the fluorophore-labelled precursor solution applied from the edges and then photopatterned at 20 mW cm⁻² using the projection SLA system described and characterized in ESI,† Section S1 and Fig. S2, S3. Actuators positioned the sample to enable 24 different exposure regions with exposure durations of 7.5 s to 25 s in 2.5 s increments, 30 s, and 35 s. Because the most significant change in mechanical properties was achieved after a single in-swelling and exposing cycle in Fiedler *et al.*,²¹ only a single cycle was used.

To confine the experiment to one dimensional transport for comparison to the model, the samples were exposed with a uniform rectangular photopattern (1.03 mm \times 1.99 mm), effectively reducing the study to one infinite region of exposure and one infinite region of darkness when probing along the pattern edge. Features were patterned using three exposure intensities $(I_0 = \{5, 10, 20\} \text{ mW cm}^{-2})$ to investigate the relationship between one-dimensional pattern fidelity and I_0 . To probe if the hydrogel chemistry exhibited reciprocity, where equivalent energy doses $E_{\rm d}$ (exposure time $t_{\rm exp}$ multiplied by I_0) result in equivalent degree of polymerization, each intensity was used at a range of doses (E_d = {20, 50, 100, 150, 200, 250, 300, and 400} mJ cm⁻²). Immediately following photopatterning, the samples were placed in a bath of DI water for 24 hours to remove unreacted monomer and finally stored in a light-proof container to prevent the fluorophore photobleaching.

Two-dimensional photopattern fidelity was probed using the same patterning technique discussed above, changing only the photopattern. The photopattern was changed to include a range of feature widths (10 μ m to 210 μ m) and feature separation distances (20 μ m to 100 μ m). The varied widths and separation distances in the pattern explored both the resolution of the photopatterning system and the effect that feature proximity has on adjacent feature development.

Confocal fluorescence microscopy

The samples were imaged on a confocal microscope (Zeiss LSM 5 Pascal system using a Zeiss Axiovert microscope) using a $10\times$ water immersion objective. The collected fluorescence intensities were then used to determine the effective concentration of attached thiol monomer species and the modulus using the techniques described in ESI,† Section S1 and Fig. S4–S8 employing the methods developed by Fiedler *et al.*²⁶

Atomic force microscopy

Atomic force microscopy (AFM, Asylum Research MFP 3D Classic AFM) was used to determine the elastic modulus of the photopatterned hydrogels. AFM was conducted using two modes, one to probe the hydrogel elastic modulus and the other to image the surface topography. These two modes combine to produce a representative map of the hydrogel, allowing direct comparison of the interplay between elastic modulus and the corresponding surface topography.

Contact mode AFM was used to image the photopatterned hydrogel surface topography,²⁷ whereas force volume mapping

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(FVM) was used to determine the elastic modulus of the photopatterned hydrogels.²⁸ This technique requires the cantilever to physically detect the surface and apply a specified force on the sample. The cantilever detects the repulsive force as a function of deflection, which was converted into indentation depth, using a position sensitive detector, producing a force versus indentation curve.²⁹ With soft (10 kPa to 100 kPa) adhesive hydrogels, it is critical to ensure the cantilever sufficiently releases from the sample surface to obtain valid force curve information. With a constant trigger force of 25 nN, the average indentation depth into the hydrogel samples was governed by sample stiffness and varied between 1 μ m and 2 µm. The force curves were analyzed through a fitting routine using the Hertz model to extract the elastic modulus.³⁰ 90 μ m imes90 µm AFM scans were digitally combined to produce a 90 µm \times 1550 µm measurement of the photopattern. The image scansize for the topographic mapping in contact mode was 90 μ m imes90 μ m (128 pixels \times 128 pixels probed). A narrower scan size was used for FVM (25 μ m \times 90 μ m) to decrease total probing time and the pixels probed decreased to 8 pixels by 32 pixels resulting in a 25 μ m \times 1550 μ m stitched image.

To obtain a cross-sectional profile of the pattern modulus, all pixels along the vertical, *y*, axis were averaged. Due to low signal to noise ratio that arose from surface variability and contaminants (*e.g.*, air bubbles, delaminated hydrogel, *etc.*) the cross-sectional data were processed through a Savitsky–Golay digital smoothing filter.³¹ This filter was chosen to enhance the signal to noise ratio without greatly affecting the signal.

Modelling photopatterned structure fidelity

Traditional SLA employs photopolymerization to locally gel a liquid resin, limiting the final material to a single mechanical property when fully crosslinked. However, high fidelity structures are challenging to achieve in high water content hydrogels due to high mobility of the monomers and propagating species during polymerization. These species diffuse until they become immobile through reaction with the solid crosslinked network. To enable photo-tunable mechanical properties and highfidelity structures, the work of Fiedler et al.²¹ was exploited. Our previously reported technique employs a hydrogel formed off-stoichiometry and then swollen with the same resin that formed the initial gel. This approach enables tunable mechanical properties while providing a reactive solid network for monomer attachment that reduces diffusive transport due to pendant species and increased crosslink density, and therefore enhances structure fidelity.²¹

In this section, the equations for the two primary limits to patterning fidelity are derived using established reaction kinetics for thiol:norbornene polymerizations. These primary limits define the critical dimension (*i.e.*, the smallest patternable feature) and the resolution (*i.e.*, the smallest gap between polymerized features). The former was controlled by the monomer diffusion from dark regions into illuminated regions. The latter was controlled by diffusion of propagating radicals from the illuminated region into the dark region. Mathematical expressions are derived to predict these two characteristic limits that define feature size.

The off-stoichiometry of this system ensures the concentration of pendant and mobile reactive groups are of similar order and thus mobile species attach to the gel after only a few reactions. The characteristic diffusion distance of these species before attachment to the network was approximated by a firstorder model in which transport distances are limited by a single reaction. The accuracy of this approximation increases as the concentration of pendant groups increases with respect to the concentration of mobile species.

Photopolymerization kinetics

Here we derive approximate expressions for the critical transport distances, starting with a kinetic model of the thiolnorbornene polymerization. Reddy *et al.*²⁰ and Cramer *et al.*³² modeled and experimentally verified the polymerization kinetics of the thiol–ene step growth polymerizations, which are used herein. The polymerization involves the addition of a thiyl radical to a vinyl functional group *via* propagation (k_p) (R1), followed by radical chain transfer (k_{CT}) from the radical carbon produced in R1 to a thiol functional group (R2):

(propagation)

$$\mathbf{RS}^{\bullet} + \mathbf{R}'\mathbf{CH} = \mathbf{CH}_2 \xrightarrow{k_p} \mathbf{R}'\mathbf{C}^{\bullet}\mathbf{H} - \mathbf{CH}_2\mathbf{SR}$$
(R1)

(chain transfer)

$$\mathbf{R}'\mathbf{C}^{\bullet}\mathbf{H} - \mathbf{C}\mathbf{H}_{2}\mathbf{S}\mathbf{R} + \mathbf{R}''\mathbf{S}\mathbf{H} \xrightarrow{k_{\mathrm{CT}}} \mathbf{R}'\mathbf{C}\mathbf{H}_{2} - \mathbf{C}\mathbf{H}_{2}\mathbf{S}\mathbf{R} + \mathbf{R}''\mathbf{S}^{\bullet},$$
(R2)

where R", R' and R are non-reactive groups of the molecules, C=C and SH are the radical-sensitive functional groups vinyl and thiol, respectively, with C[•] and S[•] indicating the radical carbon and thiyl species, respectively. These, combined with traditional polymerization initiation and termination steps, govern the kinetic behavior of the system. Utilizing the governing equations for thiol–ene click reactions established by Cramer *et al.*³² combined with the termination kinetic relationships developed by Reddy *et al.*,²⁰ the pseudo-steady state concentrations of the carbon-centered [C[•]] and the thiyl radicals [S[•]], at steady state, as derived in ESI,† Section S2, are

$$\left[\mathbf{C}^{\bullet}\right]_{\mathrm{s}} = \frac{R_{\mathrm{p,s}}}{k_{\mathrm{P}}[\mathrm{SH}]},\tag{1}$$

and

$$\left[\mathbf{S}^{\bullet}\right]_{\mathrm{s}} = \frac{R_{\mathrm{p,s}}}{k_{\mathrm{P}}[\mathrm{C}=\mathrm{C}]},\tag{2}$$

where the subscript 's' denotes steady state and $R_{p,s}$ is the steady state polymerization rate. Equipped with these steady state concentrations, a model to predict the fidelity of photopatterned structures was developed using known diffusion and kinetics for the specific monomers used in this study.

Diffusion characteristic distance

The fluorescence detection method described in ESI,† Section S1 and Fig. S4-S8, quantifies the tethered thiol species concentration after any untethered species have been removed by washing. At an intensity step boundary (i.e., photopattern edge), there are two cases of interest. First, thiyl radicals can diffuse out of the illuminated region and react with tethered 'ene' functionality on the original matrix network in the dark. The thiyl concentration was 10⁶ times lower than the tethered norbornene concentration of the original network, so this reaction was safely treated as first order in mobile thiyl concentration. Second, the thiol monomer can diffuse from the dark unpatterned region and react with the tethered carbon-centered radicals in the light-patterned region. Carboncentered radical concentration was maintained at steady state by the illumination pattern and, thus, was also considered be a first order reaction in mobile thiol concentration. The mathematical description of both processes is identical, so here we derive the behavior of the latter.

The steady-state diffusion and first-order reaction of the mobile thiol with the immobile network of carbon-centered radicals in one dimension can be described as,

$$0 = D_{\rm SH} \frac{\mathrm{d}^2[\mathrm{SH}]}{\mathrm{d}x^2} [\mathrm{SH}] - k_{\rm P} [\mathrm{SH}] [\mathrm{C}^{\bullet}]_{\rm s}, \qquad (3)$$

where *x* is distance from the illumination edge, D_{SH} is the diffusion coefficient for the thiol monomer and k_p is the reaction rate constant between diffusing thiol and tethered carbon-centered radicals.³³ This equation can then be solved in closed form for species [SH] assuming a constant flux through the boundary *x* = 0,

$$[SH] = [SH_{x=0}]e^{\frac{-x}{\sqrt{D_{SH}/k_{\rm P}[C^{\bullet}]_{\rm s}}}}.$$
(4)

It follows that the characteristic distance (d_c) for the thiol monomer over which it diffuses before reacting is

$$d_{\rm c} = \sqrt{D_{\rm SH}/k_{\rm P}[{\rm C}^{\bullet}]_{\rm s}}.$$
 (5)

This result can be used to make quantitative predictions regarding the distance over which mobile thiol monomers propagate into the illuminated photopattern and mobile thiyl radicals propagate into the dark before reacting in either case. The distance at which [SH] and [S•] falls off to 1/e from a one-dimensional edge is equal to

$$d_{\rm cSH} = \sqrt{\frac{D_{\rm SH}}{k_{\rm P} [{\rm C}^{\bullet}]_{\rm s}}} \tag{6}$$

and

$$d_{\rm cS^{\bullet}} = \sqrt{\frac{D_{\rm S^{\bullet}}}{k_{\rm P}[{\rm C}={\rm C}]}},\tag{7}$$

respectively.

The two most important conclusions from this model are the distinctly different characteristic diffusion–reaction distances for

radical species, S[•] and C[•] (on the order of nanometers), *versus* unreacted species, SH and C—C (on the order of micrometers) (ESI,† Section S1 and Fig. S9). The distance varies between the radical species and unreacted species because the steady-state thiyl radical concentration within the pattern was low with respect to the total concentration of unreacted species outside of the photopattern. The concentration of monomer species diffusing into the photopattern was comparable to the attached radical species within the pattern due to the chosen stoichiometric ratio. The two distances will impact different aspects of pattern fidelity.

The thiyl radical characteristic diffusion distance enforces two limits on the fidelity of the patterned structure. First is the resolution – formally the smallest printable gap between two polymerized lines. When the width of a dark gap is comparable to or smaller than d_{cs^*} , thiyl radicals diffuse out of both illuminated regions into the gap, causing unwanted polymerization and filling in the gap. The radical diffusion distance into the dark also limits the critical dimension of the printed structure – formally the narrowest printable line. If d_{cs^*} is comparable to or greater than the illuminated line width, the polymerized linewidth will be dominated by radical diffusion into the dark regions, not the illumination dimension.

The diffusion distance of thiol monomer into the illuminated region, d_{cSH} , limits the uniformity of the conversion within the polymerized feature. As shown below, monomer diffusing into the illuminated region accumulates in a characteristic distance d_{cSH} inside the illuminated edge, locally increasing the polymer concentration. Thiol monomer diffusion thus limits control over polymer concentration within characteristic distance d_{cSH} , while radical diffusion limits the critical dimension to be at least d_{cS} . Notedly, the former could be partially corrected by applying appropriate gray-scale illumination, while the latter is a more stringent limit of the material system.

Due to the low ratio of thiyl radical species diffusing out of the pattern to the total unreacted species concentration, the characteristic distance radicals can diffuse before terminating was negligibly affected by incident intensity variations. To substantiate this hypothesis, eqn (6) and (7) can be similarly derived for [C=C] and [C[•]]_s as $d_{cC=C}$ and $d_{cC^{\bullet}}$, respectively. ESI,† Section S1 and Fig. S9 compares the characteristic distances for typical patterning intensities of $I_0 = \{5, 10 \text{ and } 20\} \text{ mW cm}^{-2}$ and typical concentrations of norbornene and thiol functional groups in high water content hydrogels (0.035 M and 0.07 M, respectively). Further, because $R_{p,s}$ and thus $[C^{\bullet}]_{s}$ are proportional to $\sqrt{I_{0}}$, d_{c} is predicted to increase by a fourth-root with increased intensity $(d_{\rm c} \propto \sqrt[4]{I_0})$. This relationship indicates that recording intensity has little impact on structure fidelity. However, the respective distances [C==C] and [SH] propagate out of the photopattern before reacting are 1000 times greater than the distances $[C^{\bullet}]_{s}$ and $[S^{\bullet}]_{s}$ propagate out of the photopattern before terminating. These drastically different characteristic diffusion distances could lead to further degradation of photopattern fidelity.

We note, due to the fast diffusivity of all species within the hydrogel matrix, the relationship between exposure time and intensity is hypothesized to not be reciprocal beyond the biomolecular termination steady state discussed previously. Reciprocity is often an erroneously assumed relationship between t_{exp} and I_0 where equivalent energy doses E_d (exposure time t_{exp} multiplied by I_0) result in equivalent degree of polymerization.³⁴ Because the polymerization time scales allow monomer species to continuously diffuse into the photopattern during patterning, increasing the exposure time is expected to increase the polymer concentration within the photopattern even if dose is held constant.

Reaction-induced linear swelling strain

An additional feature of patterned hydrogels that limits photopattern fidelity is the high concentration of water and low concentration of solids content that produces reaction-induced differential swelling when spatially polymerized. For the PEG-NB/PEG-SH hydrogel presented here, the volumetric swelling ratio (*Q*), the ratio between the equilibrium swollen state of the hydrogel and a secondary state of interest, is proportional to matrix crosslink density to the -3/5 power.³⁵ The increased crosslink density in the photopatterned regions of the hydrogel thus reduces the swelling ratio of these regions with respect to the initial hydrogel network.

The higher crosslink density and thus lower swelling ratio of the patterned region causes the patterned region to shrink.²¹ This distorts printed features and, if shrinkage is simultaneous with exposure, blurs the edges of the pattern. This distortion is controlled by two swelling ratios: (1) the volume ratio between the precursor-swollen initial hydrogel network and the unswollen initial network (Q₀) and (2) the ratio between the precursor-swollen photopatterned network and the precursor-swollen initial network (Q_P), both of which were previously measured in bulk samples.²¹

The cube-root of each swelling ratio yields the linear swelling strain (λ) and allows for one-dimensional comparison of the precursor-swollen hydrogel λ_0 and the photopatterned hydrogel $\lambda_{\rm P}$.³⁶ The ratio of these two swelling strains, $\lambda_{\rm P}/\lambda_0$, yield the predicted swelling strain percent change expected at the edges of the photopattern. The swelling strain percent quantifies how the patterned hydrogel is predicted to deform at the edges with respect to the original photopattern dimensions, where cross-linking within the photopattern is assumed constant. This model also assumes that the photopatterned feature in question is sufficiently spatially isolated from adjacent photopatterns such that their contributions are negligible.

Results and discussion

One-dimensional (1D) photopattern fidelity characterization

To compare the model results to experiment, a rectangular photopattern was projected into the precursor-swollen hydrogel for the range of intensities and exposure times noted previously. Profiles of the fluorescence intensity, calibrated to thiol concentration in the gel, were taken through the center of the photopattern along the *x*-axis (green dashed box) and represent the average of 100 pixels along the *y*-axis (Fig. 3). Plotting the thiol

concentration as a function of exposure time and intensity, three features of note are observed in the photopatterned hydrogel. The first feature, Feature I, is the greater thiol concentration $([SH_p])$ inside the photopattern edge compared to the thiol concentration in the precursor solution $([SH_0])$ (Fig. 3a and c). The second feature of note, Feature II, is the rapid decline in thiol concentration as a function of distance from the photopattern edge (Fig. 3a and d). The third and final feature, Feature III, is the location of maximum concentration being some distance inside the photopattern edge (Fig. 3a and e). Each feature experimentally demonstrates the presence of the reaction kinetics, diffusion, and swelling processes described.

Feature I: thiol monomer characteristic transport distance (d_{cSH})

Feature I, a local increase of thiol concentration just inside the photopattern edge visible in Fig. 3a, c, d, is a direct result of the transport distance dcSH derived previously. Late in the exposure, when a majority of the thiol monomer in the illuminated region is consumed, there is a strong concentration gradient across the pattern edge.

This gradient drives diffusion of thiol monomer into the illuminated region where it reacts with pendant carboncentered radicals. The characteristic distance of the diffusion, d_{cSH} , can be found by setting the reaction rate equal to the diffusion rate over this distance. The development of excess polymer at illuminated edges is seen in other photo-patterned systems such as holographic photopolymers.³⁷

The predicted thiol monomer characteristic distance can be compared to the measured full-width half max (FWHM) of the thiol concentration peaks (Fig. 3b and ESI,† Section S1, Fig. S4). Once the peaks develop, the amplitude increases but the FWHM, or d_{cSH} , remains nearly constant, which is consistent with the first-order assumption that relevant properties including diffusivity and [C[•]] are approximately unchanging.

As $d_{\rm cSH}$ is predicted to be $\propto \sqrt[4]{I_0}$, varying exposure intensity does not provide a practical means to reduce the size of the patterned feature (Fig. 3b). To verify this, the experimentally determined d_{cSH} was compared to the modeled d_{cSH} . Documented values for thiol:norbornene termination coefficient ($k_{\rm t}$ = 3 \times $10^8 \text{ M}^{-1} \text{ s}^{-1}$) and the documented values for the diffusion coefficient of similarly-sized PEG molecules diffusing through a comparably crosslinked network ($D_{SH} = 85 \ \mu m^2 \ s^{-1}$) were employed.^{38,39} The propagation and chain transfer coefficient is the sole fit parameter in the model at a value of $k_{p} = k_{ct} =$ $1.3 \times 10^{6} \text{ M}^{-1} \text{ s}^{-1}$, which is well within the range documented in the literature ($k_{\rm p}$ = $k_{\rm ct}$ between 1 imes 10⁵ M⁻¹ s⁻¹ and 3.1 imes10⁶ M⁻¹ s⁻¹).^{20,23} The standard deviation of the FWHM of the thiol concentration peak at varied exposure intensities and exposure times fell within or just outside of 90% confidence bounds of the modeled characteristic distance a thiol monomer diffuses into the photopattern before reacting (Fig. 3b). While the analysis shows that increased uniform pattern intensity is not a practical way to reduce or eliminate these concentration peaks, approximate reduction of intensity within d_{cSH} of the pattern edge could be used to flatten the concentration profile using grayscale photopatterning.



Fig. 3 (a) Confocal fluorescent microscope images displaying the original unsaturated image, the saturated image, and then an image of the original and saturated images overlaid. The green dashed box indicates the region where the fluorescence was taken and averaged. The overlaid images demonstrate the movement of the edge of enhanced thiol concentration (dotted line) moving away from the photopattern edge (dashed line). (b) Full-width half max (FWHM) of measured diffusion/reaction distance as a function of increased incident $I_0 = \{5, 10, and 20\}$ mW cm⁻² plotted with the model and its 90% confidence bounds. Error bars indicate the standard deviation of the averaged confocal section. (c-e) Thiol concentration in the precursor solution plotted as a function of distance across the sample for varied exposure time t and for (c) $I_0 = 20$ mW cm⁻², (d) $I_0 = 10$ mW cm⁻², and (e) $I_0 = 5$ mW cm⁻². Each color represents the same amount of energy delivered to the photopattern across each sample, where dashed cyan = 50 mJ cm⁻², magenta = 100 mJ cm⁻², royal blue = 150 mJ cm⁻², red = 200 mJ cm⁻², green = 250 mJ cm⁻², black = 300 mJ cm⁻², and cyan = 400 mJ cm⁻². The solid horizontal lines indicate the concentration of thiol in the initial formulation and the dashed vertical line indicates the boundary of the photopattern, where everything left of the line was exposed to 405 nm light under the indicated intensities.

The magnitude of the excess [SH] depends on intensity and exposure time for doses below saturation, consistent with the hypothesis that Feature I is caused by a coupling of reaction and diffusion kinetics and is therefore not a simple function of local energy dose (Fig. 3c–e and ESI,† Section S1, Fig. S10). For equivalent, low doses, the thiol concentration at the photopattern edge is nearly inversely related to illumination intensity (Fig. 3c–e and ESI,† Fig. S10, Section S1). Thus, while optical intensity recording cannot effectively control the width of Feature I, it does influence the magnitude.

Feature II: thiyl radical characteristic distance (d_{cS})

The short d_{cS^*} predicted is observed as Feature II where the thiol concentration falls immediately beyond the edge of the photopattern. The ratio of the size of Feature I to Feature II is a square root relationship and is approximately 1000 for this resin. This is a general feature of photopolymerization and

indicates that Feature II, which controls patternable resolution and critical dimension, will always be much smaller than Feature I. Because the concentration of non-radical species outside of the photopattern is three orders of magnitude higher than the radical species concentration generated within the photopattern, radicals diffusing outside of the pattern will always travel a short distance before reacting compared to non-radical species. The thiol–norbornene reaction produces a particularly short radical species characteristic transport distance, predicted here to be $d_{cS^*} \approx 50$ nm, illustrating that highly reactive monomers are well suited to high resolution DLP (Fig. 3c–e).

Feature III: reaction-induced linear swelling strain

Because the characteristic transport distances of both the monomer and radical species are at the scale of microns or less, swelling effects dominate the loss of fidelity in this

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patterning system, captured as Feature III (Fig. 3). The decrease of volume swelling ratio, *Q*, with conversion causes differential swelling strain such that the polymerized feature is smaller than the illumination area. This effect is readily observed in Fig. 3a where two confocal fluorescent microscopy images of the photopatterned hydrogel taken at two detector gains are compared. The original image highlights the swelling strain. However, when the detector is saturated in the other image, the short transport species transport distances is highlighted. An overlay of the two images highlights the swelling strain because the thiol concentration does not align with the photopattern edge and is instead shifted inside of the photopattern boundaries, indicating matrix shrinkage (Fig. 3a).

Using the linear swelling strain formula presented previously combined with the data from bulk hydrogels fabricated using the same formulation and experimental conditions the predicted linear swelling strain ratio for this pattern is obtained.²¹ Taking the ratio between the swelling strain of an unswollen bulk hydrogels (λ_0) and the precursor-swollen, polymerized bulk hydrogels (λ_P), the expected linear swelling strain ratio within the patterned structure is $\lambda_P/\lambda_0 = 0.96$.²¹ Given this ratio and the photopattern half-width (0.99 mm), the photopatterned hydrogel is predicted to shrink 39 µm from the pattern edge. This prediction agreed within 20% of the swelling strain observed for all experimental conditions, which ranged between 30 μ m and 50 μ m. Notedly, this model does not account for increased crosslinking density at the photopattern edges as indicated by the [SH] peak nor did it account for the glass-constrained surfaces inhibiting true 3D diffusion. These simplifications are likely causes for the variation between the predicted and observed photopattern swelling strain.

Method to produce high fidelity, uniform hydrogel structures

Two mechanisms to avoid the build-up of species at the photopattern edges are (1) to reduce the total species conversion and/or (2) to design the precursor solution components to have significantly different diffusion and polymerization rates. Though thiol and norbornene are highly reactive, the low concentration of total species leads to increased time to reach gelation and allows diffusion to occur during polymerization. For high water-content hydrogels, swelling strain will produce predictably deformed structures that can be compensated for by modifying the projected photopattern to account for the swelling strain mismatch. If these experiment and formulation modifications are made, hydrogel structures can be photopatterned with resolution and fidelity limited only by the diffusion reaction transport distance, which is on the order of hundreds of nanometers.



Fig. 4 (a) Attached thiol concentration as a function of exposure time. The orange line represents the initial concentration of thiol, $[SH]_0$, in the precursor solution. (b) Heat-map displaying increasing thiol concentration (yellow = high, blue = low) as a function of exposure time and distance across the photopattern. (c) Florescence image of hydrogel at t_{exp} = 22.5 s to the (d) bitmap photopattern with the green dashed line indicating where the fluorescence profile was taken for (a) and (b) and represented an average of 100 pixels.

Multi-feature photopattern fidelity characterization

To probe the dynamic effects between adjacent photopatterned structures, hydrogels were exposed to a photopattern with varied widths and separation distances using an illumination intensity of 20 mW cm⁻² for all exposures (Fig. 4). Line widths in the pattern were (210, 105, 52, 26, 13, 10) μ m at two spacing distances of 39 μ m (left) and 78 μ m (right). This pattern is designed to probe both the critical dimension and the resolution of the hydrogel patterning system.

As with the 1D characterization experiment, Features I, II, and III are clearly observed across the multi-feature photopattern. However, the most dominant observation in these patterns is that the Features develop at distinctly different exposure times and regions of the pattern. For example, the $[SH]_p > [SH]_0$ component of Feature I is observed after $t_{exp} = 25$ s for the outermost photopattern edge, but investigating the patterned regions deeper inside the resolution pattern yield observations of Feature I after only $t_{exp} = 20$ s. Where enhanced [SH] at the pattern edge occurs simultaneously when $[SH]_p > [SH]_0$ for the 1D case, the multi-feature patterning case introduces further complexity because enhanced [SH] is visible 15 s before any region in the pattern satisfies $[SH]_p > [SH]_0$.

The divergence of when and where the Features are observed in the multi-feature case as compared to the 1D case can be understood by investigating the dimensions of the structures within the photopattern. Because the 39 μ m spacing between half of the lines in the photopattern (leftmost 5 lines) are on the order of the characteristic diffusion distance ($d_{cSH} = 30 \ \mu m$), adjacent lines depleted the dark region of unreacted PEG-SH resulting in both a less observable Feature I and polymerization between the lines. This leads to an elevated concentration of attached PEG-SH in the dark region due to depletion of [C==C] with exposure.

Conversely, the rightmost six lines in the photopattern are separated by nearly twice d_{cSH} and thus akin to the 1D case where Feature I is more pronounced and the dark line separation region has less PEG-SH attachment than the lines separated by 39 µm (Fig. 4). In principle, the relationship between maximum thiol concentration, photopattern differential swelling, and the size and proximity of photopattern can be exploited to fully control the spatial distribution of thiol concentration and thus mechanical properties. Future experiments will test if such improved control can be achieved.

Enhanced complexity of the multi-feature case aside, the scale-lengths of each Feature from the 1D case still hold true. For example, Feature III, informed by linear deformation, is observed immediately after exposure at the outermost photopattern edge and was similar to the 1D case at a distance $\sim 30 \,\mu\text{m}$ inset from the pattern edge. The thiol attachment beyond this edge, Feature II, was negligible within the resolution of the confocal microscope, consistent with the prediction of 50 nm transport distance (Fig. 4a and b). Additionally, this system demonstrates pixel-limited resolution ($\sim 10 \,\mu\text{m}$) as demonstrated by the saw-tooth pattern along the photopattern edge



Fig. 5 (a) Graph depicting the elastic modulus (kPa) of the photopatterned hydrogel ($t_{exp} = 25$ seconds, I = 20 mW cm⁻²) where (blue) is informed by AFM and Savitsky–Golay filtered and (orange) is informed by confocal fluorescence depicting the thiol concentration profile overlaid to highlight the enhanced modulus locations. (b) Segment of the bitmap photopattern that was used to photopattern the (c) hydrogel imaged using confocal fluorescent microscopy. Where (d) is the stitched topography images of the photopatterned hydrogel with the scale bar indicating 100 μ m. Also an image of stitched AFM data, (e) is the hydrogel elastic modulus.

representing the diagonally oriented projector pixels, where $t_{\rm exp} = 25$ s at $I_0 = 20$ mW cm⁻² (ESI,† Section S1 and Fig. S11). As predicted by the one-dimensional photopatterning analysis, high-fidelity photopatterning was achievable using this SLA system due to the short diffusion-reaction distance (~50 nm) of the thiyl radicals, which preserve the photopattern edge. This result is important because it is the first demonstration of patterning low solids content hydrogels (10% (g g⁻¹)) at the 10 µm length-scale without the addition of viscosifiers or cytotoxic concentrations of photoinitiator.

Mechanical characterization

Increased thiol concentration is hypothesized to lead to increased cross link density and hence increased elastic modulus. To test this hypothesis, photopatterns exposed for 25 s were probed using contact mode and FVM AFM to map the material response to varied exposure times. The expected modulus was calculated using the technique use for the 1D system was then plotted with the experimentally determined modulus of each sample (Fig. 5). Increased elastic modulus was observed within the photopattern regions of the hydrogel, which matches the predicted modulus within 5% to 50% as calculated from pattern feature sizes (Fig. 5a).

The variation in predicted *versus* observed modulus is hypothesized to be due to the linear swelling strain gradient induced by increased crosslinking at the photopattern edges, which this study does not account for as it was assumed constant. This result demonstrates the ability to locally increase the elastic modulus of low-solids-content hydrogels by five times using a single swelling/photopatterning cycle and is the first validation of locally enhanced elastic modulus in hydrogels using a single photopatterned precursor solution.

The modulus irregularity for each sample is depicted in the elastic modulus maps shown in (Fig. 5e), where the dark, randomly arranged spots correspond to small surface contaminants on the photopattern surface that do not represent the true patterned modulus. The presence of these contaminants can be minimized by gently washing the sample prior to testing using purified de-ionized water.

Additionally, the topographic features of the photopatterned hydrogel demonstrate a correlation between feature separation and photopattern swelling, which translates into feature height (Fig. 5d). A buckling effect was observed between adjacent patterns where monomer was depleted the most. It is hypothesized that the differential swelling that occurs in these low solids content hydrogels causes the varied topography measured. These buckled regions have similar modulus to the baseline, unpatterned regions and appear to correlate with the line separation and linewidth, which is the subject of future work.

Conclusion

In summary, we successfully photopatterned hydrogels with locally enhanced mechanical properties using a single precursor solution formulation. A quantitative confocal fluorescence microscopy technique quantified attached thiol concentration and was applied to samples illuminated with variable intensity and exposure times. Three distinct features were observed in the thiol concentration distribution: (I) patterned thiol concentration exceeded that of the original concentration in the hydrogel precursor, (II) the thiol concentration rapidly fell off beyond the photopattern edge (~ 50 nm), and (III) the enhanced thiol concentration at the photopattern edge was shifted inside of the photopattern edge. These observations were then compared to the models to predict the concentration of attached species and differential swelling across the photopattern as a function of exposure time and intensity. This comparison showed good agreement with the three hypotheses regarding the physical cause of each Feature, informing future work to fabricate fully-defined photopatterned hydrogels structures. Finally, combining the hydrogel bulk properties with the known printed species concentration, this work experimentally validates a model to predict the modulus across the photopattern using atomic force microscopy elastic modulus measurements across the photopatterned hydrogel. This comparison qualitatively validated the use of the predictive model and the single-precursor-solution technique to fabricate locally-defined, variable modulus hydrogels. This work demonstrates photopatterned hydrogels with a factor of $5 \times$ elastic modulus control and is the first demonstration of $\sim 10 \ \mu m$ resolution digital light processing (DLP) photopatterning using a low solids content resin (10% solids by weight) without the use of viscosifier or cytotoxic levels of photoinitiator $(>0.5\% \text{ g g}^{-1})$.⁴⁰ By engineering the resin to accommodate for the swelling and characteristic diffusion distances presented here, hydrogel structures with resolution well below 10 µm are patternable. To probe the cytocompatibility of the oxygen-rich, multi-step photopatterning process employed here, further studies with cell-laden precursor solution are required. DLP is a promising technique to fabricate variable modulus materials, and further research that probes a range of patternable biomaterials is the focus of future work.

Conflicts of interest

There are no conflicts to declare.

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