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Synthesis and Orthogonal Photopatterning of Hyaluronic Acid Hydrogels with Thiol-Norbornene Chemistry

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Abstract

The patterning of chemical and mechanical signals within hydrogels permits added complexity towards their use as cell microenvironments for biomedical applications. Specifically, photopatterning is emerging to introduce heterogeneity in hydrogel properties; however, currently employed systems are limited in the range of properties that can be obtained, as well as in decoupling mechanical properties from changes in chemical signals. Here, we present an orthogonal photopatterning system that utilizes thiol-norbornene chemistry and permits extensive hydrogel modification, including with multiple signals, due to the number of reactive handles accessible for secondary reaction. Hyaluronic acid was functionalized with norbornene groups (NorHA) and reacted with di-thiols to create non-toxic hydrogels with a wide range of mechanical properties. For example, for 4 wt% NorHA at 20% modification, hydrogel mechanics from ~1 kPa up to ~70 kPa could be obtained by simply changing the amount of crosslinker. By limiting the initial extent of crosslinking, NorHA gels were synthesized with remaining pendent norbornene groups that could be reacted with thiol containing molecules in the presence of light and an initiator, including with spatial control. Secondary reactions with a di-thiol crosslinker changed mechanical properties, whereas reaction with mono-thiol peptides had no influence on the gel elastic modulus. This orthogonal chemistry was used sequentially to pattern multiple peptides into a single hydrogel, demonstrating the robustness of this system for the formation of complex hydrogels.

Keywords

Hyaluronic Acid; Biomaterials; Hydrogel; Patterning; Biomechanics

1. Introduction

Hydrogels have become a ubiquitous tool for bioengineers since their properties mimic features of the extracellular environment and can provide structure and molecule delivery to control cell behavior [1,2]. Hydrogels have evolved into complex materials where they present temporally and spatially controlled mechanical properties, ligands, degradation, drug elution, and topography to cells [3–6]. This enhanced complexity better mimics the natural behavior of the extracellular matrix and can be used to investigate heterogeneous environments, such as during development and in disease states. To address questions

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related to cells and their microenvironment, it is important to independently alter only one property, so that the impact of that feature on cells can be decoupled from other signals. Creating a hydrogel with the flexibility to spatiotemporally alter multiple properties independently is not trivial and requires the careful selection of chemistry that permits both gel formation and subsequent modification.

Photopatterning is one method to alter the properties of a hydrogel monolith as it provides both spatial and temporal control since the desired reaction only occurs when and where light is applied [3]. Furthermore, light based multi-photon reactions provide a means to pattern features within a hydrogel without altering its surface properties, unlike 2D patterning techniques such as wrinkling and soft lithography [7,8]. In the simplest form, such systems use the same chemistry (e.g., light induced radical polymerization of (meth)acrylate functionalized macromers) to form the initial gel, as well as to photopattern peptides, proteins, or small molecules containing reactive groups [9]. These traditional radical-(meth)acrylate systems are straightforward, but the concentration of crosslinks and peptides are coupled within the photopatterned regions. Thus, systems to photopattern hydrogels such that crosslink density and peptide/molecule modifications can be altered independently are needed.

Orthogonal systems are useful for the formation and modification of hydrogels, where the reactions used have high specificity and reactivity toward a particular functional group. These systems typically employ a spontaneous gelation reaction (i.e., the components react when mixed), including azide-alkyne [10–13], Diels-Alder [14,15], and tetrazine-norbornene [16] cyclo-additions, as well as thiol-ene Michael additions [17–23] and condensation reactions [24, 25]. After gelation, the above systems can be designed to permit photopatterning. For example, light sensitive nitrobenzyl ether moieties allow for cleavage of chemical groups in gels to reduce gel modulus, introduce topography, or release molecules [6,13,26]. Radical generation with light sensitive initiators has also been used to pattern additional crosslinks in gels, temporally increasing mechanical properties and controlling proteolytic degradation, but these systems are limited only to changing the mechanics of gels [17–20,22]. To pattern proteins and peptides, coumarin-protected thiols can be exposed with light to expose thiols, which are then further reacted to pattern proteins and other molecules [15,24]. Finally, the radically initiated thiol-ene reaction between thiols and (meth)acrylates has been used to pattern ligands, but due to the high reactivity of the (meth)acrylates to radical polymerization they polymerize as well, changing mechanical properties while patterning ligands [10–13,27,28].

In contrast, the radically induced thiol-norbornene click reaction alleviates the concerns of non-specific reactions due to the high reactivity of thio-radicals to norbornene and the low reactivity of norbornene-radicals to norbornenes [27,28]. This light induced thiol-norbornene reaction has been used extensively to form hydrogels [29–37], but few approaches have utilized this chemistry to photopattern hydrogels [16]. These thiol-norbornene hydrogels have been made exclusively from multi-arm (4–8) PEG macromers that are end functionalized with norbornene groups. In a typical system, three of the norbornene functional groups are required to form a network, leaving only one norbornene per macromer on average for further functionalization of peptides, proteins, and small molecules. As a result, these systems are limited in the range of functionalization possible during patterning of mechanics or chemical ligands.

Here, we addressed the limitations of previous orthogonal photopatterning approaches by developing a simple but robust system using the thiol-norbornene chemistry to form and photopattern hydrogels. To facilitate this goal, hyaluronic acid (HA) was used as the macromolecular backbone as it is not only part of the native extracellular matrix, but it has

numerous pendent groups along its backbone that are accessible for functionalization with norbornene.

2. Materials and methods

2.1. General material and methods

All chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise stated. Sodium hyaluronic acid (NaHA) with a M_n of 90 kg/mol was purchased from Lifecore Biomedical. Tetrabutylammonium hydroxide (TBA-OH) and glass cover slips were purchased from Fisher Scientific and used as received. Syringes for gel formation (1 mL) were purchased from BD Biosciences. Cell adhesion peptide with the sequence GCGYGRGDSPG (RGD) was purchased from Genscript. Phosphate buffered saline (PBS), peptide synthesis resin, and fluorenylmethyloxycarbonyl (Fmoc) protected peptides were purchased from Life Technologies. An Omnicure 1000 UV light source filtered to 320–390 nm was used for gelation and photopatterning. Laser scanning confocal microscopy was performed on Leica SP5 and Zeiss LSM 510 microscopes. Proton nuclear magnetic resonance (^1H NMR) spectroscopy was performed on a 360 MHz Bruker DMX 360. Statistical significance was found using single-factor ANOVA with $p = 0.5$.

2.2. Synthesis of norbornene modified hyaluronic acid (NorHA)

Prior to NorHA synthesis, HA was converted to its tetrabutylammonium salt (HA-TBA) so that it would be soluble in dimethyl sulfoxide (DMSO). To make HA-TBA, NaHA was dissolved in deionized water (DI H_2O) at 2 wt% and the Dowex 50W proton exchange resin was added to the solution (3 g resin per 1 g NaHA) and allowed to exchange for 5 h. The resin was filtered off and the filtrate was titrated to a pH of 7.03 with TBA-OH (~1.4 TBA per HA repeat unit). The resulting solution was frozen at -80°C , lyophilized, and stored at -20°C until used. See Figure S1 for the ^1H NMR spectrum of HA-TBA.

To synthesize NorHA, HA-TBA was dissolved in anhydrous DMSO (2 wt%) with a 3:1 molar ratio of 5-norbornene-2-carboxylic acid (mixture of endo and exo isomers) to HA-TBA repeat units and 4-(dimethylamino)pyridine (1.5 molar ratio to HA-TBA repeat units) was added under a N_2 atmosphere. The solution was heated to 45°C and di-*tert*-butyl dicarbonate (Boc_2O) was added by syringe into the flask at a 0.4 molar ratio to HA-TBA repeat units. After 20 h, cold water was added to quench the reaction and the solution was purified by dialysis for 3 d to remove DMSO. After dialysis, NaCl was added (1 g NaCl per 100 mL of solution) and the solution was precipitated into 10-fold excess cold (4°C) acetone. The precipitate was dissolved in DI H_2O , frozen at -80°C , lyophilized, and stored at -20°C until used. The product was analyzed by ^1H NMR spectroscopy (Figure S1) and the NorHA was found to have ~20% of its repeat units functionalized with norbornene. ^1H NMR shifts of pendent norbornenes (D_2O) : 6.33 and 6.02 (vinyl protons, *endo*), 6.26 and 6.23 (vinyl protons, *exo*), 3.20–2.90 (bridgehead and protons), and 1.57–1.27 ppm (bridge and ring protons).

2.3. Synthesis of fluorescent and thiol containing peptides

Using standard solid supported (glycinol 2-chlorotriyl resin) Fmoc protected peptide synthesis, peptides with the sequence GCDDD-Fluor were synthesized, where Fluor denotes one of three fluorescent molecules: 5(6)-carboxyfluorescein (FSH), rhodamine b (RSH), or 7-methoxycoumarin-2-acetic acid (McSH). The peptide was cleaved from the resin with trifluoroacetic acid, precipitated twice into cold diethyl ether, dissolved in PBS, frozen at -80°C , and lyophilized. All peptides were stored in the dark at -20°C until needed. Products were confirmed by ^1H NMR spectroscopy and MALDI. See Figure S2 for ^1H NMR spectra and Figures S3–S5 for MALDI spectra of the various peptides.

2.4. Fabrication of NorHA gels and mechanical testing

NorHA was dissolved in PBS to a desired concentration with varying amounts of dithiothreitol (DTT) and 0.05 wt% 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (I2959). The weight percent of NorHA (W_{NorHA}), ratio of DTT thiols to norbornenes (X_{DTT}), and irradiation time (t_{I}) were varied systematically for the gels. The prepolymer solution (50 μL) was transferred to syringes with their tips removed, covered with a No. 1 cover slip, and irradiated for the desired time with UV light at 10 mW/cm^2 power. Individual gels were removed from the syringes and placed in 1 mL of PBS to swell overnight before mechanical testing. Compressive moduli were found using a TA Instruments DMA Q800 fitted with a compression clamp, running at a constant strain rate of 10%/min in air at room temperature. The modulus was calculated from the slope of the stress-strain curve between 10 and 20% strain ($n = 3$).

2.5. Cell toxicity and encapsulation studies

For assessment of the toxicity of soluble NorHA, human mesenchymal stem cells (hMSCs) were plated at a density of 2500 cells/ cm^2 on TCPS and incubated in growth media overnight. Subsequently, the media was supplemented with NorHA at varying concentrations (0, 1.25, 2.5, and 5 mg/mL) and changed every 3 days. On days 4 and 7, metabolic activity was assessed with Alamar Blue (Invitrogen) as specified in manufacturer's instructions. Briefly, cells were incubated with 10% Alamar Blue in serum-free media for 3 hours and the fluorescence was read with 570/610 nm as the excitation/emission wavelengths. The % relative viability for NorHA containing media was calculated as the ratio of each solution's fluorescence to the average fluorescence of the Alamar Blue solution for cells cultured without NorHA.

For encapsulation studies, hMSCs were suspended in macromer precursor ($W_{\text{NorHA}} = 4$ wt %, $X_{\text{DTT}} = 0.3$, 0.05 wt% I2959) solution in PBS at 10^7 cells per mL under sterile conditions. The solution (40 μL) was transferred to sterile syringe tips, covered with a cover slip, and irradiated for 30 s with UV light (10 mW/cm^2). The gels were transferred to 24-well plates with 2 mL of hMSC growth media and incubated at 37 °C. At 1 and 3 d of incubation, gels ($n = 3$) were removed from media and stained with a LIVE/DEAD cell viability assay (Invitrogen) following the manufacturer's instructions and imaged with confocal microscopy.

2.6. Secondary modification of NorHA gels

NorHA gels were made as described above ($W_{\text{NorHA}} = 4\%$, $X_{\text{DTT}} = 0.3$ or 0.5 , $t_{\text{I}} = 30$ s) and the resulting gels were swollen overnight in PBS at 4 °C. After this wash step, the desired secondary reaction solution was introduced into the gels overnight at 4 °C and the gels were irradiated with UV light (10 mW/cm^2) for 120 s. To achieve additional crosslinking, solutions of DTT at various concentrations (C_{DTT}) with 0.05 wt% I2959 in PBS were introduced into each gel. To demonstrate that modification with a mono-thiol molecule does not alter the mechanical properties, a solution of the FSH (0.04 mM) and 0.05 wt% I2959 in PBS was introduced into a series of gels. To demonstrate that functionalizing the gels with RGD does not alter the mechanical properties, RGD (2 mM) and 0.05 wt% I2959 were introduced into the gels. All gels were compression tested as described above.

2.7. Photopatterning NorHA gels

To provide a stable surface to attach thin gels, cover slips (No. 2 glass, 22 $\times$ 22 mm) were plasma etched for 2 min, coated with (3-mercaptopropyl)trimethyl siloxane, and baked for 1 hr at 100 °C and then 10 min at 110 °C. The residue was washed from these thiolated cover slips (with dichloromethane, acetone, and methanol), dried, and stored under N_2 (no more

than 24 h) prior to use. To fabricate the gels, 100 μL of prepolymer solution ($W_{\text{NorHA}} = 4 \text{ wt} \%$, 0.05 wt% I2959, various X_{DTT}) was placed on a large cover slip (No. 1 glass, 45 by 50 mm dimensions). The thiolated cover slips were placed thiol-side down on the solution while supported by No. 2 glass cover slips at its edges and the solution was irradiated through the thiolated cover slip with UV light ($10 \text{ mW}/\text{cm}^2$) for 30 s, yielding a gel with $\sim 200 \mu\text{m}$ thickness that could be removed from the bottom cover slip while remaining attached to the thiolated cover slip.

Gels prepared as described above were incubated overnight at 4°C in the desired peptide or DTT solutions. Typical patterning solutions for fluorescent peptides were 60, 4, and 5 μM of McSH, RSH, and FSH, respectively, with 0.05 wt% I2959 and 1 wt% bovine serum albumin (BSA) in PBS. Solutions to pattern RGD and DTT consisted of 2 mM thiols (1 mM DTT, 2 mM RGD), 0.05 wt% I2959, and 5 μM FSH in PBS (as an indicator of pattern location). Gels containing the desired solution were covered with a CAD drawn photomask transparency (CAD/Art Services, Inc) and irradiated for 60 s ($10 \text{ mW}/\text{cm}^2$). Patterned gels were placed in PBS and incubated at 4°C for at least 48 h with PBS changes twice a day to remove unreacted peptide or DTT. For sequentially patterned gels, after the previously mentioned incubation period the next dye solution was introduced, photopatterned, and washed out as described above. To create a gradient patterned gel, an opaque slide was moved over the gel at a constant velocity during UV exposure after the FSH solution was introduced.

2.8. Atomic force microscopy (AFM) characterization

Thin 3D NorHA gels were synthesized as described above ($X_{\text{DTT}} = 0.3$, $t_{\text{I}} = 30 \text{ s}$, $W_{\text{NorHA}} = 4\%$). Gels were photopatterned (120 s irradiation, $10 \text{ mW}/\text{cm}^2$) with solutions of either DTT (1 mM) or RGD (2 mM) using a 100 μm parallel line photomask. FSH was patterned simultaneously with DTT or RGD for visualization of the patterned regions. AFM was performed on an Asylum Research MFP-3D AFM equipped with an inverted optical microscope. The tips used were 1 μm diameter SiO_2 beads with a 0.06 N/m cantilever spring constant (Novascan). Contact force maps were obtained such that they had patterned and unpatterned regions with two force maps obtained per gel. A Hertzian model of contact behavior was used to calculate the Young's modulus assuming a Poisson's ratio of 0.5 for each force curve of the force map [38]. Modulus values from patterned regions were normalized to the unpatterned regions of the gel to give a normalized modulus ($n = 15$).

3. Results and discussion

3.1. Synthesis of NorHA and gelation scheme

NorHA was synthesized from HA-TBA (Figure 1a) using Boc_2O coupling chemistry to couple norbornene acid to the HA backbone. The NorHA was collected in high yield after dialysis (90%) and had $\sim 20\%$ of its repeat units functionalized with pendent norbornenes. With this degree of functionalization, the NorHA had nearly fifty norbornenes per 90 kg/mol chain, presenting numerous reaction points. These pendent norbornenes can undergo a thiol-ene reaction with a di-thiol crosslinker to form a gel (Figure 1b). Ideally, only three norbornenes per polymer chain are required to form a network, leaving numerous other unreacted norbornenes in the network that can undergo secondary reactions to increase the crosslink density or couple chemical ligands (Figure 1b).

3.2 Tunability in NorHA gel properties

Due to the robustness of the thiol-norbornene chemistry, nearly any water soluble di-thiol molecule (e.g., peptide containing two cysteine residues) can crosslink NorHA to form a gel. In our study, DTT was used as the di-thiol linker to produce hydrolytically stable gels

(Figure S6). All gels were formed with UV light (320–390 nm) irradiated (10 mW/cm²) radical generation by the I2959 initiator at 0.05 wt% concentration for all experiments. The three parameters that were varied to control the compressive moduli (E_c) of the gels were the UV light irradiation time (t_i), ratio of DTT thiols to norbornene (X_{DTT}), and weight percent NorHA in the solution (W_{NorHA}).

At a X_{DTT} of 0.5 and W_{NorHA} of 4%, precursor solutions were irradiated with UV light for various t_i to observe the kinetics of gelation (Figure 2a). Upon irradiation, within seconds the solution gelled and the E_c quickly plateaued such that after 5 min the E_c was ~90% of its plateau value (20 min). Rheology (Figure S7) confirmed this fast gelation as the storage modulus (G') increased orders of magnitude in less than 10 s and it also confirmed that once irradiation ceased gelation stopped as evidenced by the unchanging modulus. Unlike other orthogonal gelation/patterning systems, the E_c of NorHA gels can be controlled simply by t_i as the reaction ceases once light is removed, whereas spontaneous reactions go to completion.

The X_{DTT} was also varied at W_{NorHA} equal to 4% and a t_i of 45 min, well past the 20 min to reach plateau E_c (Figure 2b). For X_{DTT} values of 0.1 to 0.6, the plateau E_c increased nearly linearly with increasing X_{DTT} due to increased crosslink density from the DTT reacting with the excess norbornenes off NorHA. At X_{DTT} equal to 1, an equal amount of thiols and norbornenes are present and for an ideal system each thiol would react with a norbornene to make an effective crosslink, giving a maximum crosslink density and therefore a maximum E_c . At X_{DTT} values around 1 (0.6 to 1), this maximum was observed (Figure 2b). The maximum E_c begins at a lower X_{DTT} than expected likely due to the non-ideal nature of the system where some of the DTT-norbornene reactions occurred with norbornenes on the same NorHA chain and as a result did not form an effective crosslink. With a X_{DTT} greater than one, the E_c drops from the maximum value due to excess thiols in the system saturating the norbornenes before the maximum crosslink density can be achieved. Thus, the resulting gels likely have free thiols pendent off the NorHA chains in the network.

Further demonstration of the flexibility of the E_c obtained from the NorHA system was observed by varying the W_{NorHA} at t_i and X_{DTT} equal to 30 s and 0.5, respectively (Figure 2c). By changing the W_{NorHA} an order of magnitude, the observed E_c changed two orders of magnitude. These results along with the t_i and X_{DTT} data demonstrate the tunability of this system without the need to synthesize a new macromer. Additionally, the control afforded to the system due to its initiation with light allows for specific moduli to be targeted and the possibility for further reactions after gelation.

3.3. NorHA cytocompatibility

To demonstrate NorHA's utility as a biomaterial, the cytocompatibility of the macromer with human mesenchymal stem cells (hMSCs) was studied. hMSCs were cultured with NorHA at various concentrations in growth media (0–5 mg/mL) for 7 days. After 4 days of incubation, there was no statistically significant difference in metabolic activity of the hMSCs (Alamar Blue) cultured in NorHA containing media as compared those cultured in media without NorHA (Figure S8). After 7 days, there was a reduction in the metabolic activity for cells cultured at the highest NorHA concentration (5 mg/mL). Additionally, hMSCs were encapsulated in 3D NorHA gels (W_{NorHA} = 4%, X_{DTT} = 0.3, t_i = 30 s) and incubated for one and three days. The fast gelation of this system allowed for cells to be quickly encapsulated (30 s) with low toxicity (96 and 88 % viability for one and three days after encapsulation, respectively, Figure S9), confirming the low toxicity of NorHA and suggesting its possible use for cell studies.

3.4. NorHA gel secondary modification

After gelation, we targeted secondary reactions between thiol-containing molecules and unreacted norbornenes in the gel. To ensure that norbornenes did remain after gelation, all gels to be patterned had X_{DTT} values below 1 (0.3–0.5) and a t_{I} equal to 30 s. During rheological studies, sequential irradiation ($t_{\text{I}} = 30$ s) produced step changes in G' , indicating that norbornenes remained after the initial gelation (Figure S7). ^1H NMR spectroscopy of gels degraded by hyaluronidase (Figure S10) confirmed the presence of norbornenes after the initial 30 s exposure. For a t_{I} of 30 s, the E_{c} as function of X_{DTT} still behaved as it did at t_{I} equal to 45 min (Figure 2d) and a maximum E_{c} was observed around X_{DTT} equal to 1 and gels with a range of E_{c} were produced.

NorHA gels irradiated for 30 s ($X_{\text{DTT}} = 0.3$, $W_{\text{NorHA}} = 4\%$) were used as starting materials to alter the mechanical properties after initial gelation. The initial gels were washed with PBS and a reaction solution consisting of 0.05 wt% I2959 and various concentrations of DTT (C_{DTT}) was incubated with the gel overnight. The gels were subsequently irradiated with UV light (10 mW/cm^2) for 2 min and their E_{c} was measured (Figure 3a). When a solution of only I2959 ($C_{\text{DTT}} = 0$) was reacted with the gel, no change in E_{c} from the original gel was observed. This result suggests that there are few free thiols remaining in the gel that can form additional crosslinks. Additionally, this result confirms that the norbornenes will not significantly react with each other through a radical mechanism.

Unlike adding just I2959, the addition of low concentrations of DTT with I2959 yields an increase in E_{c} until a plateau modulus is achieved around C_{DTT} equal to 2 mM (Figure 2a). This secondary crosslinking gives an over 2-fold increase in E_{c} over the initial value. The maximum E_{c} value obtained through a secondary reaction is less than the maximum E_{c} through initial gelation (46 versus 70 kPa), which could be a result of unreacted NorHA being removed during the initial swelling with PBS – nearly 15% as indicated by the aforementioned stability study (Figure S6) – before the secondary reaction.

To further demonstrate the orthogonal nature of this system, mono-thiols were reacted with NorHA gels ($X_{\text{DTT}} = 0.5$, $t_{\text{I}} = 30$ s, $W_{\text{NorHA}} = 4\%$) in a similar fashion to the DTT secondary reaction. The mono-thiols should not increase the E_{c} of the NorHA gel since they lack the di-thiol structure to make effective crosslinks. Adding a cysteine functionalized fluorescein dye (FSH) or RGD cell adhesion peptide containing a cysteine group with I2959 did not significantly ($p = 0.9$ and 0.2 for FSH and RGD, respectively) increase the E_{c} after irradiation for 2 min (Figure 3b). As expected, when DTT and initiator were reacted with the original gel, a significant increase in E_{c} occurred ($p = 6 \times 10^{-5}$). These results demonstrate that due to the low reactivity of norbornene to itself during light induced radical reactions, mono-thiol molecules can react with the gel and not change E_{c} , while di-thiols can change E_{c} . This ability to decouple mechanical and chemical modifications permits application of this gel to a range of biological studies.

3.5. Photopatterning NorHA gels with fluorescent dyes

With the success of the secondary reactions to alter NorHA gels, we investigated the ability to photopattern various dyes within a NorHA gel. The process to photopattern involved introducing the dye molecule that contained a thiol, exposing to light in the presence of an initiator through a mask, and washing out the unreacted dye (Figure 4a). Specifically, a NorHA gel ($X_{\text{DTT}} = 0.3$, $t_{\text{I}} = 30$ s, $W_{\text{NorHA}} = 4\%$) was placed in a reaction solution containing the desired thiol containing molecule and 0.05 wt% I2959 and incubated overnight. The gel now containing the thiol solution was covered with a transparency photomask and irradiated with UV light. Regions exposed to light underwent the thiol-norbornene reaction, while unexposed regions did not. The gel was then placed in PBS

(washed for several days) for the unreacted molecules to diffuse out of the gel, leaving behind a photopatterned gel.

For easy visualization of photopatterned regions of gels, several thiol contain fluorescent dyes were developed (Figure 4b) using solid state peptide synthesis. The peptide sequence contained a single cysteine residue that undergoes a thiol-norbornene reaction with NorHA and three aspartic acid residues to give a negatively charge peptide to decrease non-specific binding of the peptide to the gel, facilitating removal of unreacted dye. Due to the flexibility of solid state peptide synthesis, nearly any molecule containing a free carboxylic acid can be added to the N-terminus of the peptide. The dyes 7-methoxycoumarin-2-acetic acid, 5(6)-carboxyfluorescein, and rhodamine b were chosen to give reactive peptides that fluoresce blue, green, and red, respectively (McSH, FSH, and RSH). These dyes were individually introduced into NorHA gels with 1 wt% BSA and 0.05 wt% I2959, masked with a 100 μm stripe pattern, irradiated (10 mW/cm²) for 60 s, and washed out with PBS over several days. Confocal microscopy images of the patterned gels (Figure 4b) illustrate the ability of this process to photopattern gels. All three gels have stripes (100 $\pm$ 1 μm , n = 7) of their respective dye separated by dark unpatterned regions.

3.6. Temporal and spatial control of gel patterns

To demonstrate that the NorHA system can be modified with dyes with precise spatial and temporal control, a ~200 μm thick NorHA gel was synthesized attached to a cover slip ($X_{\text{DTT}} = 0.3$, $t_{\text{f}} = 30$ s, $W_{\text{NorHA}} = 4\%$) to provide easy handling and imaging of the gel. A solution of FSH, I2959 (0.05 wt%), and BSA (1 wt%) was introduced into the gel and a gradient photopatterning process was developed where a mask of 100 μm stripes was placed on the gel. The gel was initially irradiated for 30 s before an opaque slide was moved across the gel at a constant rate (147 $\mu\text{m/s}$), blocking the UV light. The resulting photopatterned gel had regions irradiated over the range of 30 s to 110 s with UV light. The intensity of the dye fluorescence increased with longer irradiation time and consequently with position along the gel (Figure 5). Each peak in Figure 5 corresponds to a region exposed to light where the valleys correspond to regions not exposed (see image insets). The intensity increased as more dye reacted with the gel and eventually plateaued as the rate of photobleaching of the dye caught up to the rate of addition. Longer irradiation times led to a decrease in intensity due to this photobleaching (100–110 s). The temporal control of the dye coupling further confirms that the secondary thiol-norbornene reaction can be used to control the dosage of molecules throughout the gel.

3.7. Mechanical properties of photopatterned gels

Secondary reactions to the bulk gels altered mechanics and ligand introduction (Figure 2), so we investigated whether such behavior translates to photopatterned gels. NorHA gels (200 μm thickness, $X_{\text{DTT}} = 0.3$, $t_{\text{f}} = 30$ s, $W_{\text{NorHA}} = 4\%$) were photopatterned with 100 μm stripes of either DTT (1 mM DTT, 2 mM thiols) or RGD (2 mM). FSH was included to visually identify regions photopatterned from those not patterned. The normalized modulus (normalized to the unpatterned region of the gel) of patterned and unpatterned regions was found by AFM assuming Hertzian mechanics (Figure 6). The RGD patterned gel had uniform normalized modulus throughout with no significant difference observed between the patterned and unpatterned regions. As expected, patterning DTT gave a significant 2-fold increase in the normalized modulus. Interestingly, a similar 2-fold increase in E_c was observed in the bulk gels with 2 mM DTT (Figure 3a), suggesting that the reaction is consistent for both patterned and bulk reactions. These results confirm that patterning modulus and the introduction of mono-functionalized molecules can be achieved independently.

3.8. Sequential photopatterning

NorHA gels ($X_{\text{DTT}} = 0.3$, $t_{\text{I}} = 30$ s, $W_{\text{NorHA}} = 4\%$) were sequentially patterned with each fluorescent dye to demonstrate the flexibility of the system to independently pattern several molecules into one gel. The above patterning procedure for one dye was used to pattern McSH with 100 μm diameter circles, then FSH with 100 μm per side triangles, and then RSH with 100 μm wide stripes. Imaging of the stained gel revealed that all dyes and their respective patterns were incorporated into the gel (Figure 7). Additionally, several dyes and their patterns overlapped (McSH and RSH, McSH and FSH), indicating that under the conditions investigated norbornenes remained after the first dye was patterned so that the second dye could be patterned over it. Such results suggest that several molecules can be sequentially patterned in a single region, effectively creating complex shapes of molecules.

The FSH triangle mask did have some loss of pattern size when transferred to the gel as indicated by the triangles having edges of ~ 50 μm and not 100 μm (Figure 7a). The loss of dimensions could be due to the fact the FSH triangles were patterned on top of the McSH circles. McSH has a non-zero absorbance over the range of the UV light source (320 – 390 nm) and it may have absorbed the UV light that would have been used to couple FSH to gel. The reduction in UV light may have reduced the FSH concentration reacting with the gel and therefore its overall intensity, reducing pattern size. Further evidence of such absorption by McSH can be seen in the vertical slice through the gel (Figure 7e). The FSH intensity reduces more quickly through the depth of the gel when compared to the other two dyes. Both McSH and RSH pattern through the depth of the gel (~ 200 μm , ~ 325 μm in thicker gels in Figure S11) demonstrating that this system could be used to pattern in 3D a projection of the 2D mask with multiple molecules. Also, multi-photon approaches could be used to increase the depth and complexity of the patterns.

4. Conclusions

NorHA is an easily synthesized and versatile macromer that can be crosslinked with UV light and di-thiol molecules to create gels with a wide range of mechanical properties from a single macromer. By selecting conditions where pendent norbornenes remain in the gel, orthogonal secondary reactions can be performed to the gels. The high specificity of the norbornenes to react only with thiols allowed for the photopatterning of mono-thiol molecules without changing the gel compressive modulus, as well as patterning of mechanical properties with di-thiol molecules. The robust photopatterning reaction created gels that were both spatially and temporally patterned, as well as sequential patterned materials with several different molecules and patterns. The straightforward synthesis, orthogonal patterning, and tunability of this system makes it ideal to systematically and independently study the effects of mechanical properties and molecules in biological systems.

Supplementary Material

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Figure 1.

(a) Scheme to synthesize NorHA from HA-TBA through the coupling of norbornene carboxylic acid to pendant alcohols on HA. The reaction proceeded through a di-tert-butyl dicarbonate (Boc₂O) activated process, yielding NorHA with ~20% of its repeat units functionalized with norbornene. (b) Synthesis scheme to form gels through the light initiated thiol-ene reaction between a di-thiol and NorHA with subsequent chemical modification with mono- and/or di- thiols.

Figure 2.

(a) Compressive modulus (E_c) as a function of UV light irradiation time (t_i) for a precursor solution with a 0.5 ratio of thiol to norbornene (X_{DTT}) and NorHA weight percent (W_{NorHA}) of 4%. (b) E_c as a function of X_{DTT} ($W_{NorHA} = 4\%$, $t_i = 45$ min). (c) E_c as a function of W_{NorHA} ($X_{DTT} = 0.5$, $t_i = 30$ s). (d) E_c as function of X_{DTT} at t_i equal to 30 s ($W_{NorHA} = 4\%$). All precursor solutions contained 0.05 wt% I2959 and were irradiated at 10 mW/cm² of UV light. Error bars are one standard deviation ($n = 3$).

Figure 3.

(a) E_c of NorHA gels ($X_{DTT} = 0.3$, $t_I = 30$ s, $W_{NorHA} = 4\%$) after secondary reactions with different concentrations of DTT introduced into gel (C_{DTT}) and UV light irradiation (0.05 wt% I2959, 2 min irradiation, 10 mW/cm²). The solid line represents E_c of the original gel (dashed lines indicate standard deviations). All other error bars denote one standard deviation ($n = 3$). (b) E_c of gels ($X_{DTT} = 0.5$, $t_I = 30$ s, $W_{NorHA} = 4\%$) after incubation with various thiol solutions and subsequent irradiation with UV light (2 min, 10 mW/cm²). Bars labeled dye, RGD, and DTT refer to fluorescein labeled peptide (FSH, 0.04 mM), RGD peptide (2 mM), and DTT (1 mM, 2 mM thiols), respectively. Error bars indicate one standard deviation ($n = 3$) and asterisk represents statistical significance ($p < 0.05$).

Figure 4.

(a) Schematic of process to photopattern NorHA gels with thiol containing molecules. (b) Chemical structures of fluorescent dye terminated peptides with confocal images of photopatterned (100 μm stripes mask) NorHA gels with each dye. Peptide dyes were synthesized using solid supported peptide Fmoc chemistry to give GCDDD-Fluorescent sequences. The cysteine residue in each peptide provides a thiol group for the radical initiated thiol-ene reaction.

Figure 5.

Patterned fluorescein dye (FSH) fluorescence intensity as a function of irradiation time and position on the gel with confocal images of selected regions of the patterned gel. The spatial and temporal gradient of fluorescence was generated by passing an opaque mask over a 100 μm line patterned photomask irradiated with UV light (10 mW/cm^2). Each second of irradiation corresponds to 147 μm of linear travel. Images above the plot correspond to the red outlined areas on the plot.

Figure 6.

Normalized modulus for photopatterned and non-photopatterned regions of NorHA gels, patterned with either RGD or DTT. Modulus values (Hertzian) were calculated from AFM and normalized to the unpatterned regions of each gel. Gels were patterned by irradiation with UV light (10 mW/cm^2) through a $100 \text{ }\mu\text{m}$ line photomask for 120 s. FSH was included in the patterned regions for visual confirmation of patterned regions. Average values were calculated from force mapping over two areas for each gel that were unpatterned and patterned. Error bars are of one standard deviation. *denotes statistical significance.

Figure 7.

Fluorescent confocal images of sequentially patterned NorHA gel ($X_{\text{DTT}} = 0.3$, $t_{\text{I}} = 30$ s, $W_{\text{NorHA}} = 4\%$). Images for the split channels are presented for (a) FSH triangles, (b) RSH lines, and (c) McSH circles as well as the (d) combined fluorescent channels. Image (e) demonstrates that the patterns span the depth of the gel (~200 μm).