Cytocompatible Click-based Hydrogels with Dynamically-Tunable Properties Through Orthogonal Photoconjugation and Photocleavage Reactions

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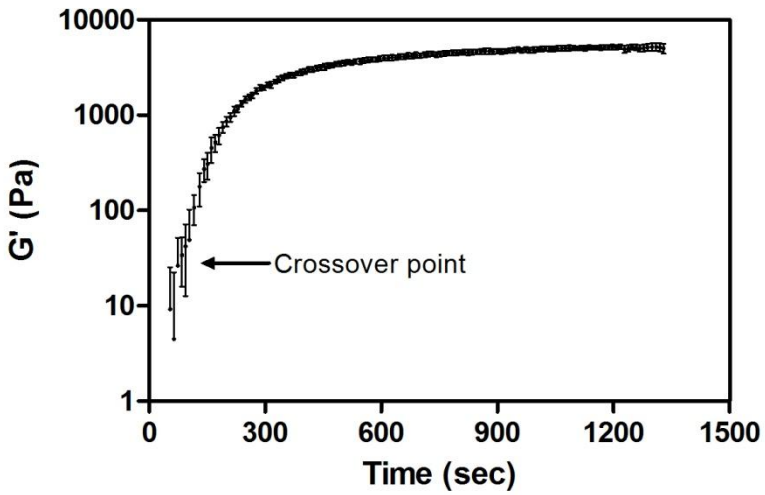
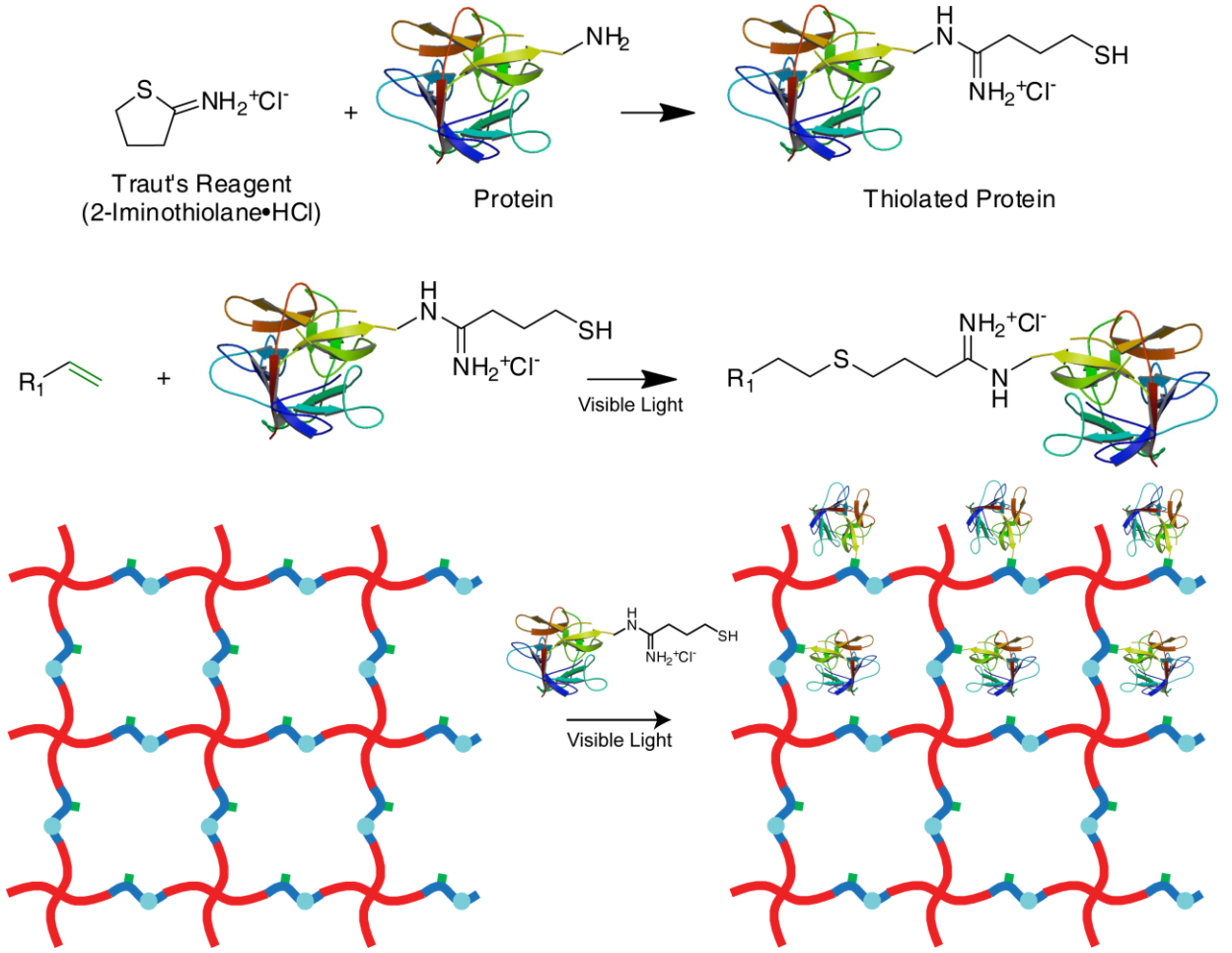
Figure S1 *In situ* rheometry of gel formation



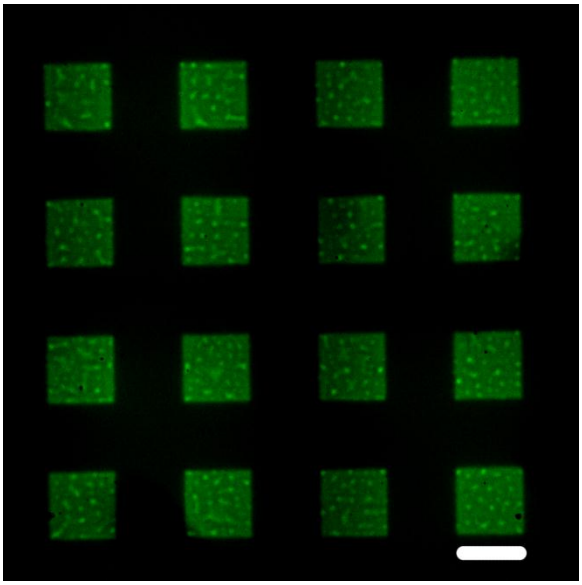
Dynamic frequency, time, and strain sweep rheological experiments were performed on a TA Ares rheometer with parallel plate geometry (8 mm diameter) at 25 °C. Initial gel network formation of a 10 wt% solution was monitored by observing G' and G'' at a constant frequency of 100 rad/s as a function of time. Gel properties were monitored *via* frequency sweep measurements at fixed strain amplitude (10%) to measure the hydrogel storage, G', and loss, G'', moduli. The crossover point was found to be at 100 ± 20 sec. Final modulus was determined to be 5100 ± 500 Pa.

Figure S2 Protein patterning within click gels



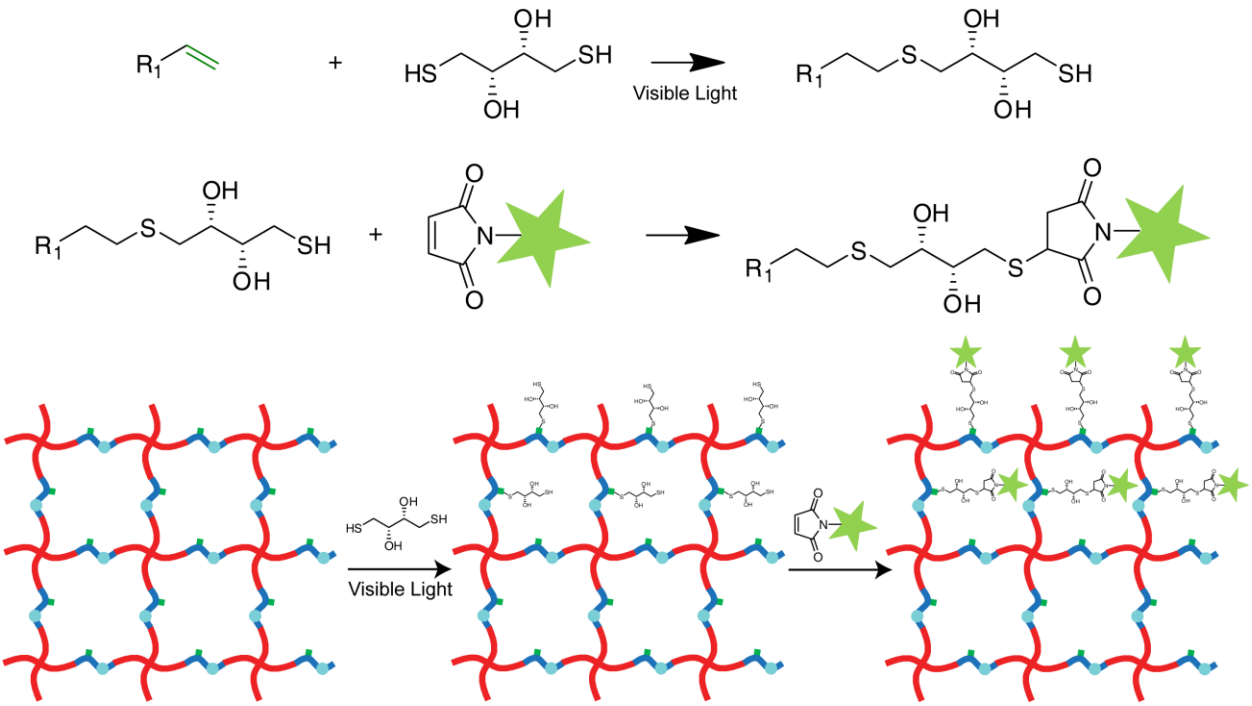
Traut's reagent (2-Iminothiolane•HCl, Thermo Scientific, 2x) was added to soybean trypsin inhibitor, Alexa Fluor® 488 conjugate (STI_{488}, 21 kDa, Invitrogen) dissolved in PBS at 0.67 mg mL^{-1} and reacted at room temperature for 1 hr to yield the thiolated protein (STI_{488}-SH). Traut's reagent reacts with primary amines on the ST_{488} and converts them to thiols to be used in the thiol-ene reaction.

Hydrogels were swollen in phenol red-free media containing STI_{488}-SH (0.1 mg mL^{-1}) and eosin Y (10 μM) for one hour. Gels were exposed to collimated visible light (λ = 490 – 650 nm), achieved with an Acticure (EXFO) high pressure mercury lamp equipped with an internal bandpass filter (350 – 650 nm) and an external 490 nm longpass filter (Edmund Optics), through a patterned chrome photomask with 200 x 200 μm square openings. Unreacted STI_{488}-SH and initiator were removed by swelling into fresh media, and the sample was visualized by fluorescent confocal microscopy (image below).



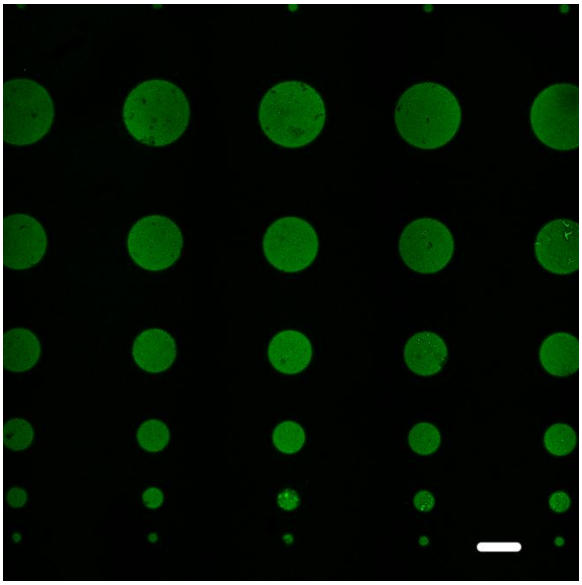
Scale bar = 200 μm.

Figure S3 Small molecule patterning within click gels



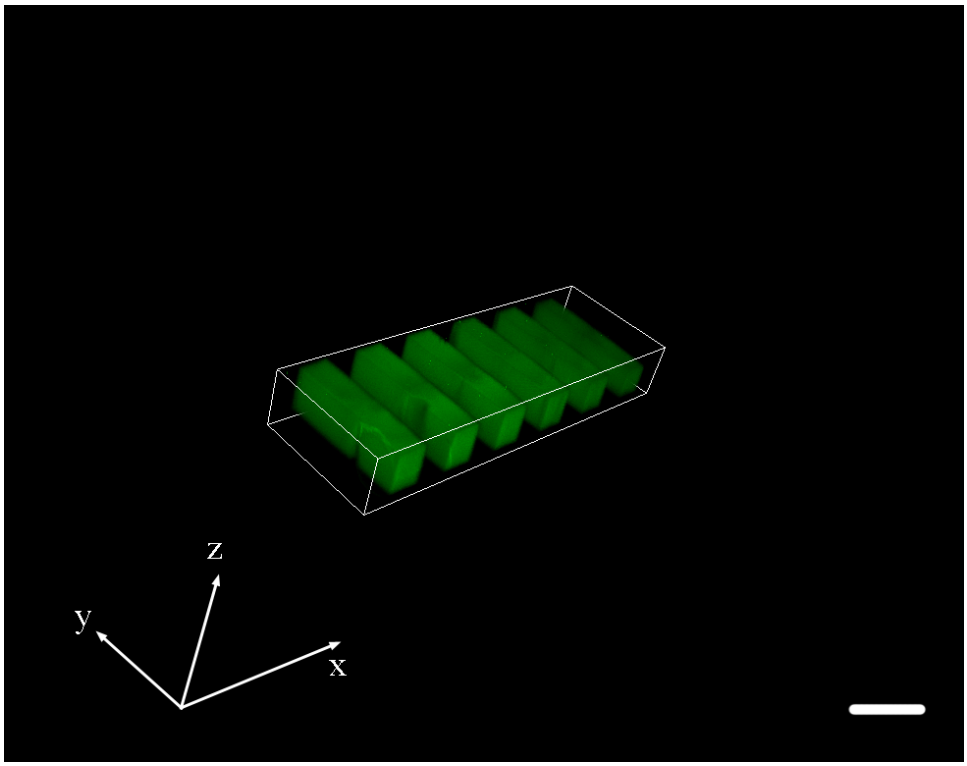
Hydrogels were swollen in phenol red-free media containing dithiothreitol (DTT, 0.3 mg mL^{-1}) and eosin Y (10 μM) for one hour. Gels were exposed to collimated visible light (λ = 490 – 650 nm), achieved with an Acticure (EXFO) high pressure mercury lamp equipped with an internal bandpass filter (350 – 650 nm) and an external 490 nm longpass filter (Edmund Optics), through a patterned chrome photomask with 50, 100, 150, 200, 250, and 300 μm diameter circle openings. Unreacted DTT and initiator were removed by swelling into fresh media, yielding the chemically-patterned hydrogel.

To visualize the patterned thiol molecules, phenol red-free media containing Alexa Fluor® 488 C5 maleimide (0.2 mg mL^{-1}) was swollen into the network. Here, the maleimides covalently react with the patterned pendant thiols, which are present only where light was previously shone. The unreacted dye was swollen into fresh media, and the sample was visualized by fluorescent confocal microscopy (image below).



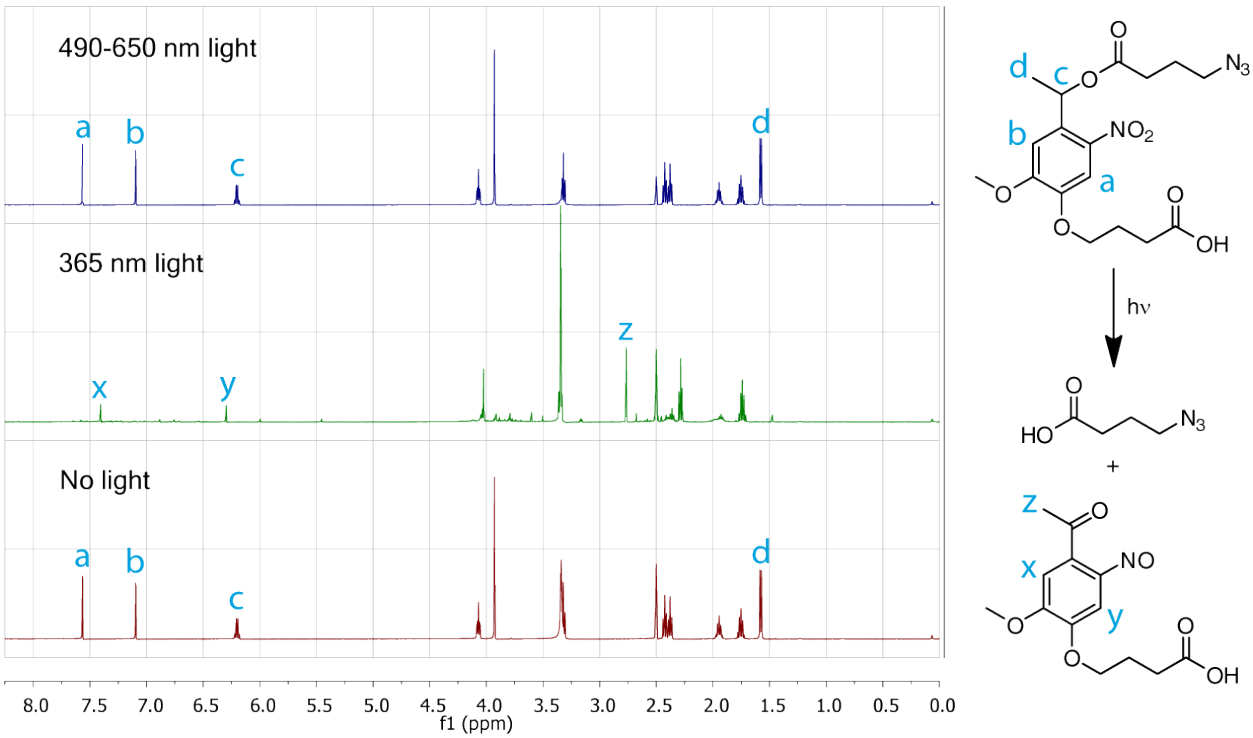
Scale bar = 200 μm.

Figure S4 3D visualization of photolithographically-based thiol-ene patterning



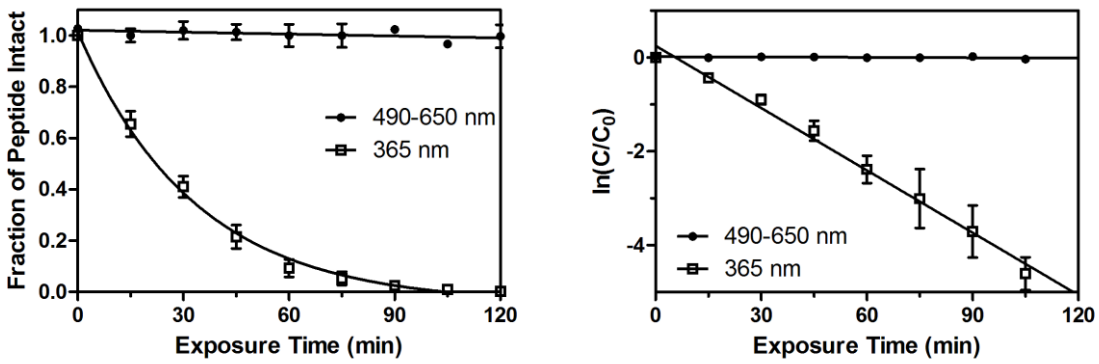
Channels are 400 μm wide (in x) and extend through the full thickness of the gel (~500 μm, in z) with roughly constant intensity. Scale bar = 800 μm.

Figure S5 NMR studies of small molecule PLazide photodegradation



Upon exposure to UV light (λ = 365 nm), characteristic peaks in ^{1}H NMR shift drastically indicating photodegradation. In similar exposure to visible light (λ = 490 – 650 nm), these peak shifts are not observed indicating negligible photodegradation.

Figure S6 Kinetic NMR studies of PLazide photodegradation



The photodegradable peptide (Azide-RGK(alloc)GRK(PLazide)-NH_2) was dissolved in dH_2O at 15.5 mM, roughly the concentration present in a 10 wt% gel formation, and injected into a glass sample chamber measuring 3" x 2" x 500 μm. The solution was exposed to collimated light (λ = 365 nm or 490 – 650 nm, 10 mW cm^{-2}) for various amounts of time. The solution was collected, lyophilized, redissolved in D_2O, and ^1H NMR experiments were performed for each time point. The fraction of intact peptide was calculated by comparing integral values for the alloc vinyl protons (δ = 5.84, 1H), which do not shift significantly upon degradation and thus give the amount of peptide present in the sample, with the aromatic protons of the intact PLazide (δ = 7.50, 1H; δ = 7.11, 1H).

The photodegradation process follows first-order reaction kinetics:

$$\frac{C}{C_0} = e^{-k_{avg} \cdot t}$$

Where C is the concentration of intact PLazide at any time t, C_0 is the initial concentration of the PLazide (15.5 mM), and k_{avg} is the kinetic constant of degradation averaged throughout the thickness of the sample. It is important to note that k_{avg} does not account for light attenuation in our specific system and is specific to each experimental setup. From this, it follows that:

$$\ln \frac{C}{C_0} = -k_{avg} \cdot t$$

Plotting our data (left) in this form (shown on the right) gives us a linear plot with a slope:

$$k_{avg} = 7.36 \times 10^{-4} \frac{1}{\text{sec}}$$

Similarly, the kinetic constant can be written in more fundamental terms:

$$k_{avg} = \frac{\varphi \varepsilon I_{avg}}{N_A \hbar \nu}$$

$$\varphi = \frac{N_A \hbar \nu k_{avg}}{\varepsilon I_{avg}}$$

Where ϕ is the quantum yield; N_A is Avogadro's number; h is the Planck constant; c is the speed of light; I is the intensity of light; I_0 is the incident light intensity; ε is the molar absorptivity of the sample (4780 M^{-1} cm^{-1} for PLazide at λ = 365 nm); and ν is the frequency of the associated electromagnetic wave.

Also, the light intensity at any given depth (z) is a function of C and ε.

$$I = I_0 e^{-\varepsilon C z}$$

As both the degradable precursor and the degraded product have similar molar absorptivities (ε) at λ = 365 nm (see Figure S4), the light attenuation is roughly constant throughout the degradation process and can be described as:

$$I = I_0 e^{-\varepsilon C_0 z}$$

We can calculate the average intensity as:

$$I_{avg} = \frac{\int_{0\ \mu\text{m}}^{500\ \mu\text{m}} I_0 e^{-\varepsilon C_0 z} dz}{\int_{0\ \mu\text{m}}^{500\ \mu\text{m}} dz} = 2.53 \frac{\text{mW}}{\text{cm}^2}$$

Thus, all variables in our equation for ϕ are known:

$$\varphi = 0.01995$$

Using this quantum yield, we can calculate the kinetic constant for degradation at any intensity, I.

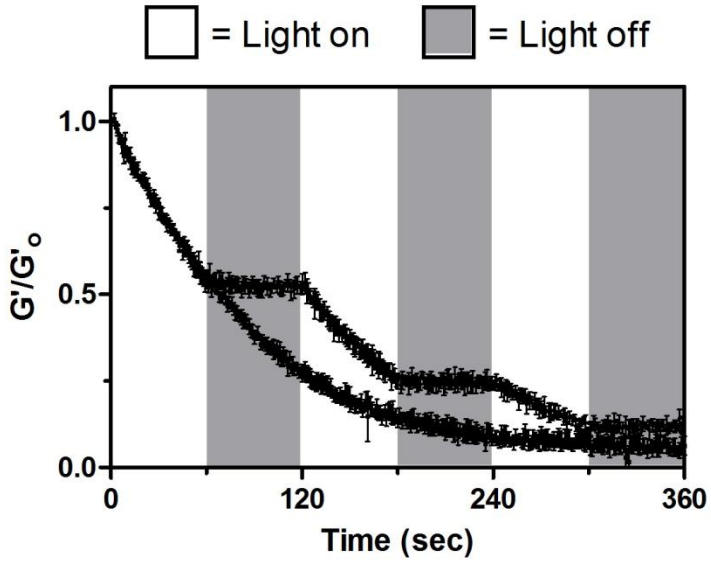
$$k = \frac{\varphi \varepsilon I}{N_A \hbar \nu}$$

For 10 mW cm^{-2}:

$$k = 0.00291 \frac{1}{\text{sec}}$$

This value, unlike k_{avg}, is intrinsic to the photodegradation reaction and is not specific to a given experimental setup.

Figure S7 Temporally controlled photodegradation and material properties of optically thin gels

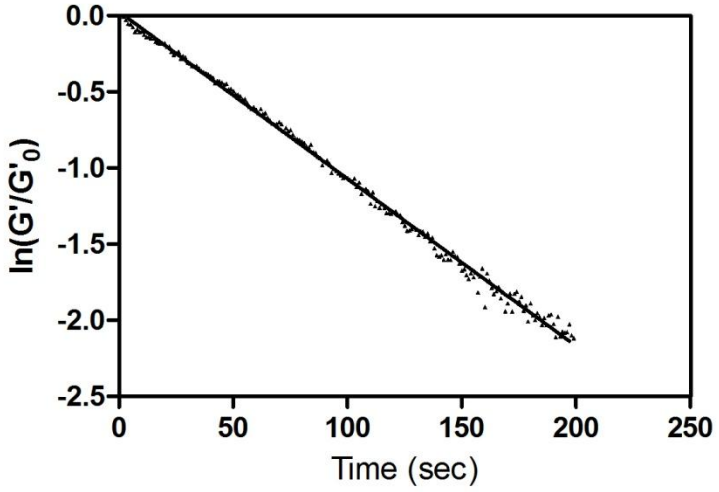


Optically thin gels (50 μm) were polymerized *in situ* between a Peltier plate (25 °C) and a clear quartz plate (8 mm) on a photorheometer (TA Ares). Dynamic time sweep experiments were performed to monitor gel formation (see Supplementary Fig. S1). After full gelation had occurred (~20 min), the sample was exposed to UV light (λ = 365 nm, 10 mW cm^{-2}) and degradation was quantified by monitoring G' with constant exposure (bottom curve). By shuttering the light exposure, temporal control over network properties was obtained (top curve).

The crosslinking density (ρ_x) scales with the measured storage modulus (G'), we can calculate the degradation kinetic constant (k) from the continuous exposure data as:

$$\frac{\ln(G')}{\ln(G'_0)} = \frac{\ln(\rho_x)}{\ln(\rho_{x,0})} = -4kt$$

Here, G'_0 is the initial storage modulus (5100 ± 500 Pa), $\rho_{x,0}$ is the initial crosslinking density, and t is the irradiation time. The factor of 4 is included as photolytic cleavage on any side of the 4-arm PEG will lead to a decrease in crosslinking density.

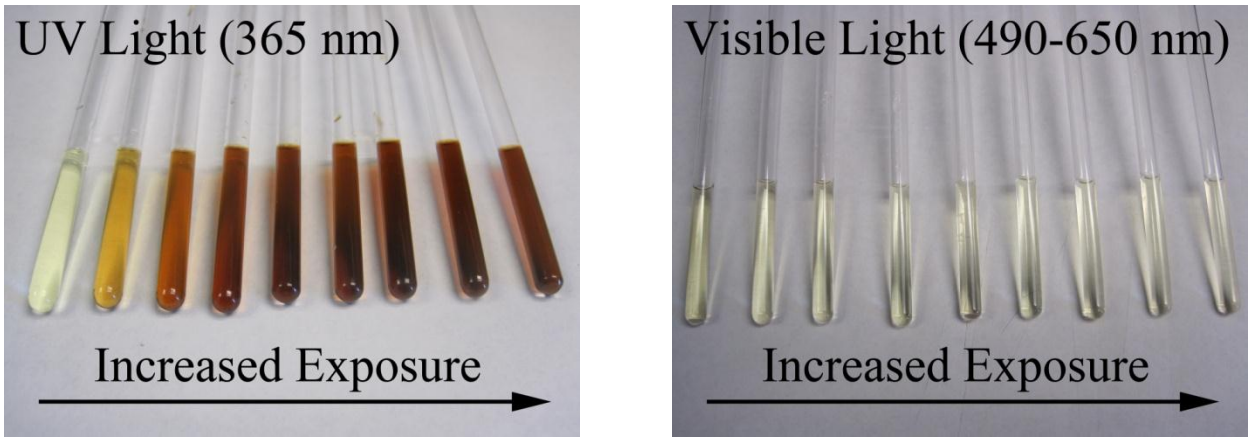


From the slope of the $\frac{\ln(G')}{\ln(G_0')}$ vs *t* plot, we obtain:

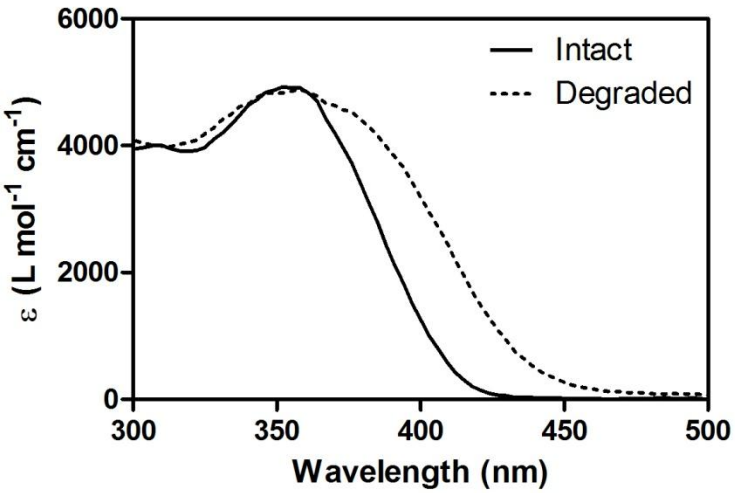
$$k = 0.00275 \frac{1}{\text{sec}}$$

which is in good agreement with that obtained by NMR studies of PLazide degradation in Supplementary Fig. S6.

Figure S8 Visible color change upon Azide-RGK(alloc)GRK(PLazide)-NH_2 photodegradation

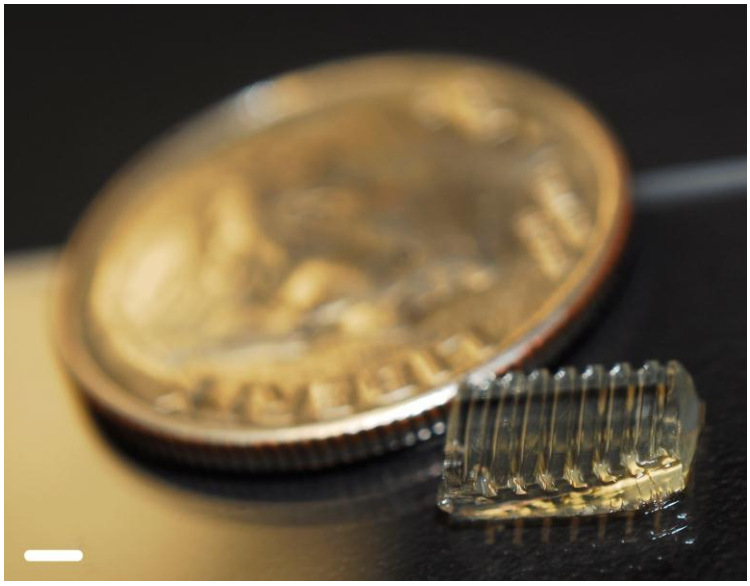


Digital photographs of Azide-RGK(alloc)GRK(PLazide)-NH_2 after light treatment (as described in Supplementary Fig. S6). Images each represent 0, 15, 30, 45, 60, 75, 90, 105, and 120 min light exposure from left to right for 10 mW cm^{-2} for λ = 365 nm and λ = 490 – 650 nm.



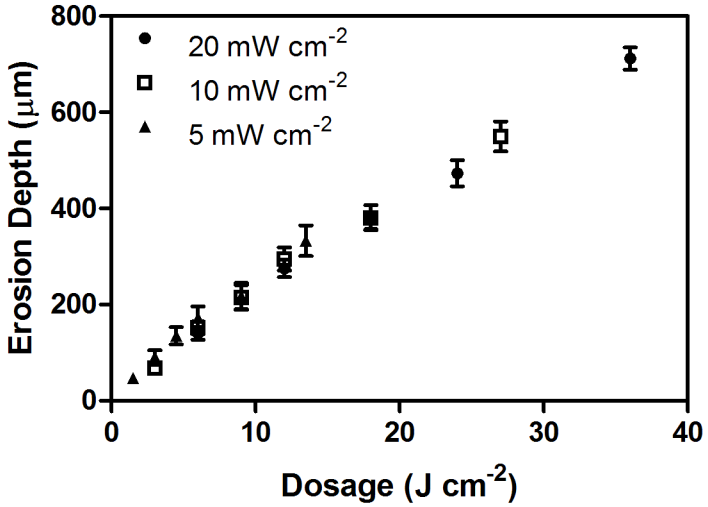
The darkening of the samples upon degradation is seen by a shift in the absorption spectrum for PLazide.

Figure S9 Digital photo of photodegraded channels in optically-thick sample



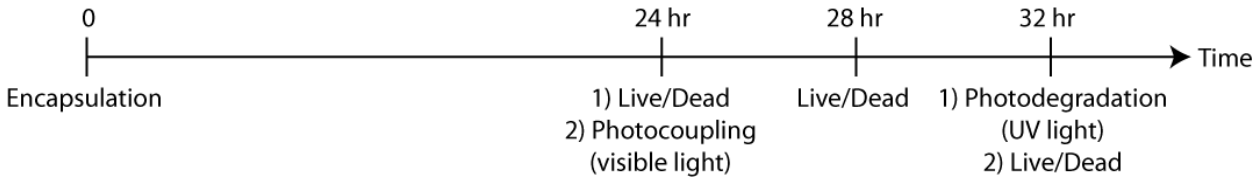
Channels are 400 μm wide and 400 μm deep. Scale bar = 1.6 mm.

Figure S10 Photodegradation erosion depth versus total light dosage



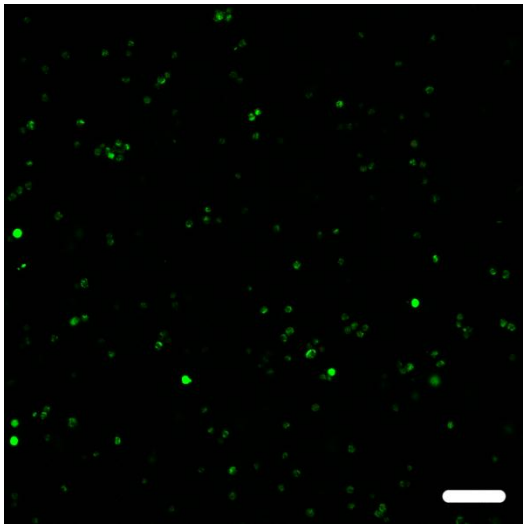
Here, dosage represents the total amount of energy delivered to the system and is calculated as the product of exposure time and light intensity. The erosion depth is measured by profilometry.

Figure S11 Viability of hMSCs after SPAAC encapsulation, thiol-ene coupling, and network photodegradation



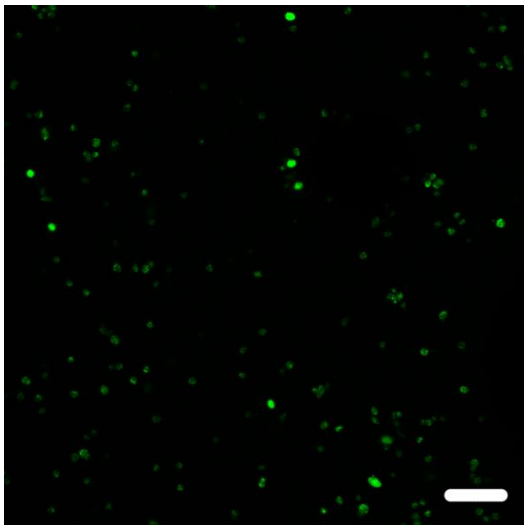
Time 0 – Human mesenchymal stem cells (hMSCs, between P3 and P6) were encapsulated in 10 wt% photodegradable, photocouplable click formulation in PBS at a density of 2.5 x 10^6 cells mL^{-1}. The solution was sandwiched between azide-functionalized (See Supplementary Fig. S15) and Rain-X®-treated glass slides spaced at 0.5 mm, and reacted for 30 min. The gels were transferred to growth media (consisting of low-glucose Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Gibco), 0.2% fungizone, and 0.4% gentamicin) and incubated in 5% CO_2 at 37 °C.

24 hrs – A subset of the cell-laden gels were stained with a Live/Dead assay (Invitrogen) and visualized with fluorescent confocal microscopy. A high viability post-encapsulation (>95%) was observed. The unstained hydrogels were then swollen in phenol red-free media containing H-RGDSC-NH_2 (3 mg mL^{-1}) and eosin Y (10 μM) for one hour. Gels were bulk irradiated (2 min) with collimated visible light (λ = 490 – 650 nm, 10 mW cm^{-2}), achieved with an Acticure (EXFO) high pressure mercury lamp equipped with an internal bandpass filter (350 – 650 nm) and an external 490 nm longpass filter (Edmund Optics). Unreacted peptide and initiator were removed by swelling into fresh media, yielding the chemically-modified hydrogel.



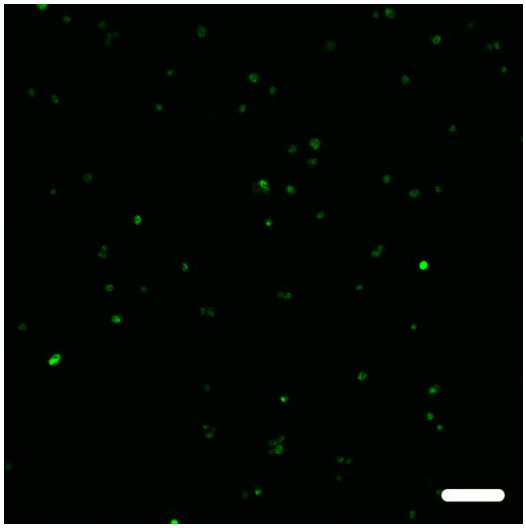
Scale bar = 100 μm, 100 μm projection

28 hr – A subset of the cell-laden, H-RGDSC-NH_2 thiol-ene labeled gels were stained with Live/Dead (Invitrogen) and visualized with fluorescent confocal microscopy, and a high viability post-encapsulation and photocoupling (>95%) was observed.



Scale bar = 100 μm, 100 μm projection

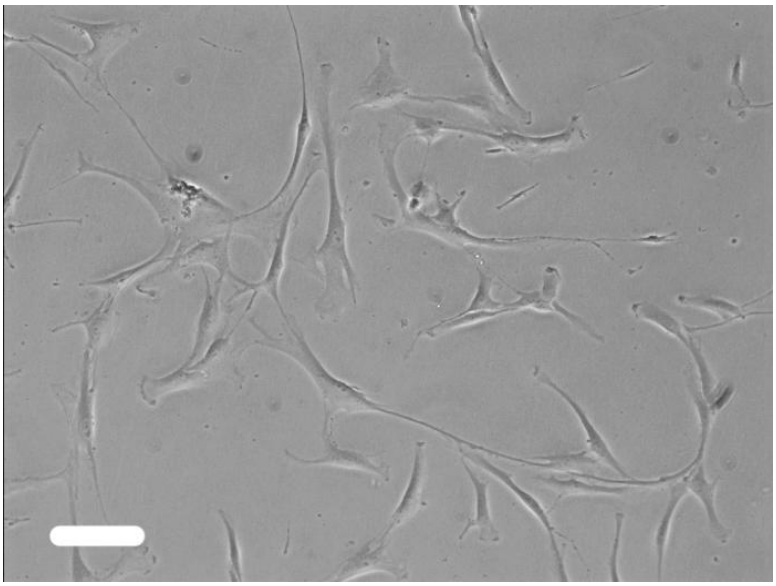
32 hr – Cell-laden gels, previously patterned with H-RGDSC-NH_2 by thiol-ene photocoupling, were bulk irradiated (15 min) with collimated UV light (λ = 365 nm, 10 mW cm^{-2}), resulting in full degradation of the top ~200 μm of the initially 500 μm thick gel. This degradation resulted in the complete release of the encapsulated cells in the eroded regions. The gel was then washed with fresh media, and the cells were concentrated by centrifugation (1000 rpm x 6 min). The cells were resuspended in media containing the Live/Dead stain (Invitrogen) for 30 min, centrifuged again (1000 rpm x 6 min), resuspended in fresh media (10^6 cells mL^{-1}), and imaged with fluorescent confocal microscopy. Again, a high viability post-encapsulation, photocoupling, and photodegradation (>95%) was observed.



Scale bar = 100 μm, 100 μm projection

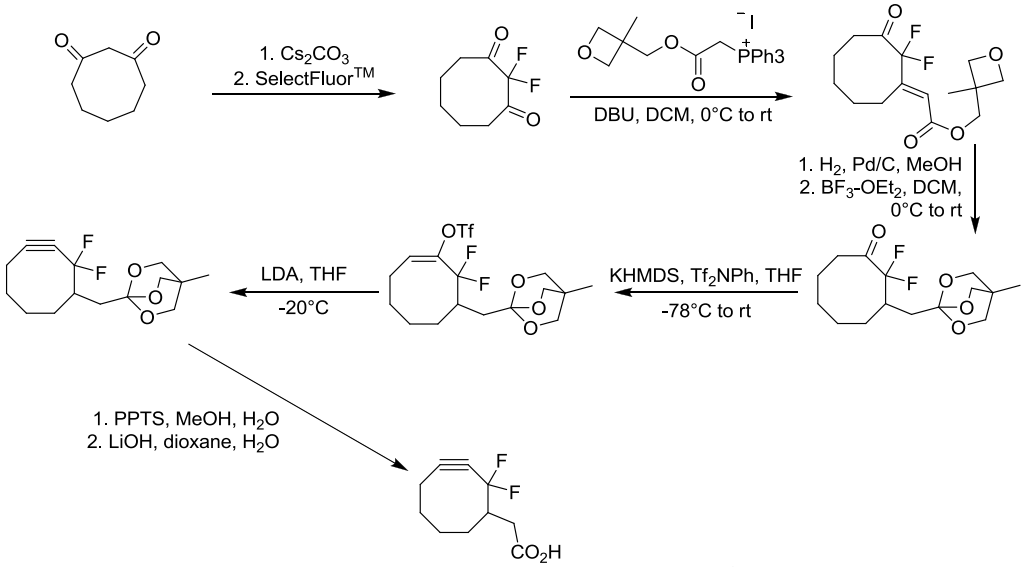
The photoreleased cells were then plated in a 96-well tissue culture polystyrene plate and imaged 48 hours later with brightfield microscopy. Cells attached and exhibited typical morphology of

plated hMSCs, further demonstrating their viability throughout the encapsulation and subsequent patterning processes.

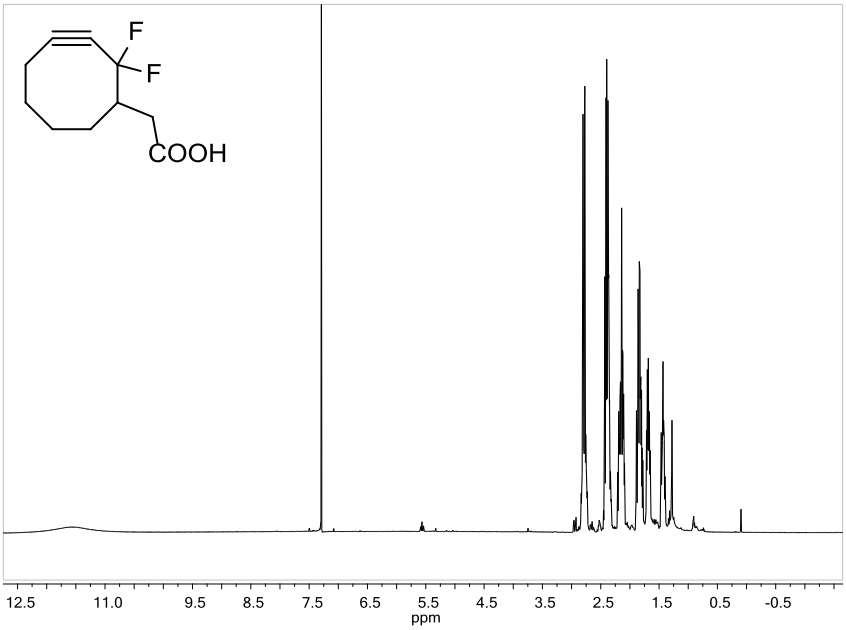


Scale bar = 25 μm

Figure S12 Synthesis of difluorinated cyclooctyne (DIFO3)



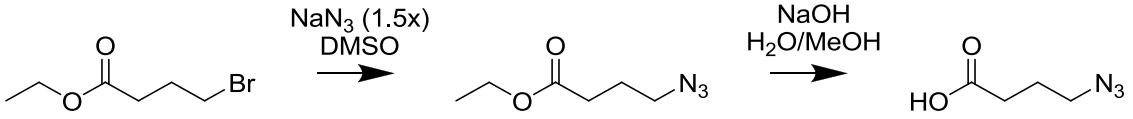
DIFO3 was synthesized following a published synthetic route[1], starting with 12.4 g of 1,3-cyclooctanedione. All reactions were performed with linearly-scaled reaction conditions with respect to reported amounts. Yields were comparable for all reported steps. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 11.72 (br s, 1H), 2.82-2.69 (m, 2H), 2.43-2.22 (m 3H), 2.19-2.05 (m, 2H), 1.87-1.74 (m, 2H), 1.70-1.61 (m, 1H), 1.45-1.35 (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$, δ): 178.53 (s), 119.10 (dd, J = 237.7, 239.4), 110.71 (t, J = 11.1 Hz) 84.62 (dd, J = 41.6 Hz, 46.9), 52.60 (t, J = 24.3 Hz), 33.80 (d, J = 3.1 Hz), 32.84 (d, J = 4.5 Hz), 32.65 (d, J = 1.9 Hz), 27.87 (s), 20.45 (s); ^{19}F NMR (376 MHz, $CDCl_3$, δ): -95.84 (d, J = 259.9; 1F), -102.05 (ddt, J = 7.0, 21.1, 260.2; 1F); HRMS (ESI+): calculated for $C_{10}H_{12}F_2LiO_2^+$ [M + ^{7}Li]$^+$, 209.0940; found 209.0965 (Δ = +2.5 ppm).



1. Codelli, J.A., Baskin, J.M., Agard, N.J. & Berozzi, C.R. Second-generation difluorinated cyclooctynes for copper-free click chemistry. *J Am Chem Soc* **130**, 11486-11493 (2008).

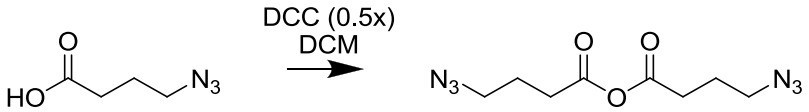
Figure S13 Synthesis of Azide-functionalized Photodegradable Precursor (PLazide)

Synthesis of 4-azidobutanoic acid:



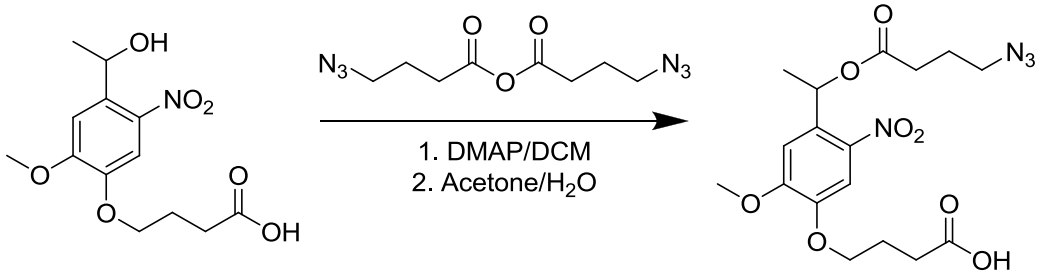
Ethyl-4-bromobutrate (100 g, 513 mmol) was dissolved in DMSO (750 mL) and stirred under argon overnight at 55 °C with sodium azide (50 g, 1.5x, 769 mmol). The crude reaction mixture was diluted with dH_2O (500 mL) and extracted into diethyl ether (3 x 500 mL). The combined organic phases were washed with water (500 mL), brine (500 mL), dried over $MgSO_4$, filtered, and concentrated to yield 80.5 g (512 mmol, quantitative yield) of ethyl 4-azidobutanoate. This intermediate was dissolved in a mixture of 1 N NaOH (500 mL) and methanol (300 mL) and stirred at room temperature for 3 h. The methanol was then removed by rotary evaporation, and the aqueous phase's pH was brought to 0 with dropwise addition of HCl, and the product was extracted into diethyl ether (3 x 500 mL). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated to yield 64.9 g (502 mmol, quantitative yield) of the 4-azidobutanoic acid product. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.64 (br s, 1H), 3.34 (t, 2H), 2.44 (t, 2H), 1.88 (p, 2H); ^{13}C NMR (101 MHz, $CDCl_3$, δ): 50.46, 30.92, 23.94; HRMS (ESI+): calculated for $C_4H_6N_3O_2^-$ $[M - {}^{1}H]^-$, 128.0485; found 128.0462 (Δ = -2.3 ppm).

Synthesis of 4-azidobutanoic anhydride:



4-azidobutanoic acid (25.7 g,199 mmol) and N,N'-Dicyclohexylcarbodiimide (DCC, 13.2 g, 64 mmol) were purged with argon, dissolved in anhydrous DCM (160 mL), and stirred at room temperature for 45 min. The dicyclohexylurea byproduct was removed *via* celite filtration. The crude mixture was redissolved in DCM (30 mL), concentrated, and filtered, and was repeated until no urea was observed.

Synthesis of PLazide:



4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy]butanoic acid (Hydroxyethyl photolinker, EMD Novabiochem, 4 g, 13.4 mmol) and 4-Dimethylaminopyridine (DMAP, 80 mg, 0.65 mmol) was added to the anhydride mixture, dissolved in minimal DCM (100 mL) with pyridine (1.08 mL, 13.4 mmol), and stirred under argon overnight. The crude mixture was washed with aq. $NaHCO_3$ (100 mL), 1 N HCl (100 mL), and brine (100 mL). The combined organics were

dried over $MgSO_4$ and concentrated. This mixture was then dissolved in a 50:50 mixture of acetone/dH_2O (1400 mL) and stirred overnight, upon which acetone was removed *via* rotary evaporation, and the product was extracted into DCM (700 mL). The organic layer was washed with 1 N HCl (500 mL), brine (500 mL), dried over $MgSO_4$, filtered, concentrated under reduced pressure, and purified by flash chromatography (5:1 to 1:1 hexanes/EtOAc with 1% acetic acid) to yield a yellow solid (5.05 g, 12.3 mmol) in excellent yield (92%). ^{1}H NMR (500 MHz, DMSO, δ): 12.18 (s, 1H), 7.57 (s, 1H), 7.10 (s, 1H), 6.21 (q, 1H), 4.07 (t, 2H), 3.93 (s, 3H), 3.32 (t, 2H), 2.4 (p, 4H), 1.95 (p, 2H), 1.76 (p, 2H), 1.58 (d, 3H); ^{13}C NMR (101 MHz, DMSO, δ): 173.98, 171.56, 153.53, 146.89, 139.66, 131.87, 108.66, 108.42, 67.94, 67.48, 56.25, 49.87, 30.70, 29.92, 23.98, 23.72, 21.30; HRMS (ESI+): calculated for $C_{17}H_{21}N_4O_8^-$ $[M - {}^{1}H]^-$, 409.1368; found 409.1363 (Δ = -0.5 ppm).

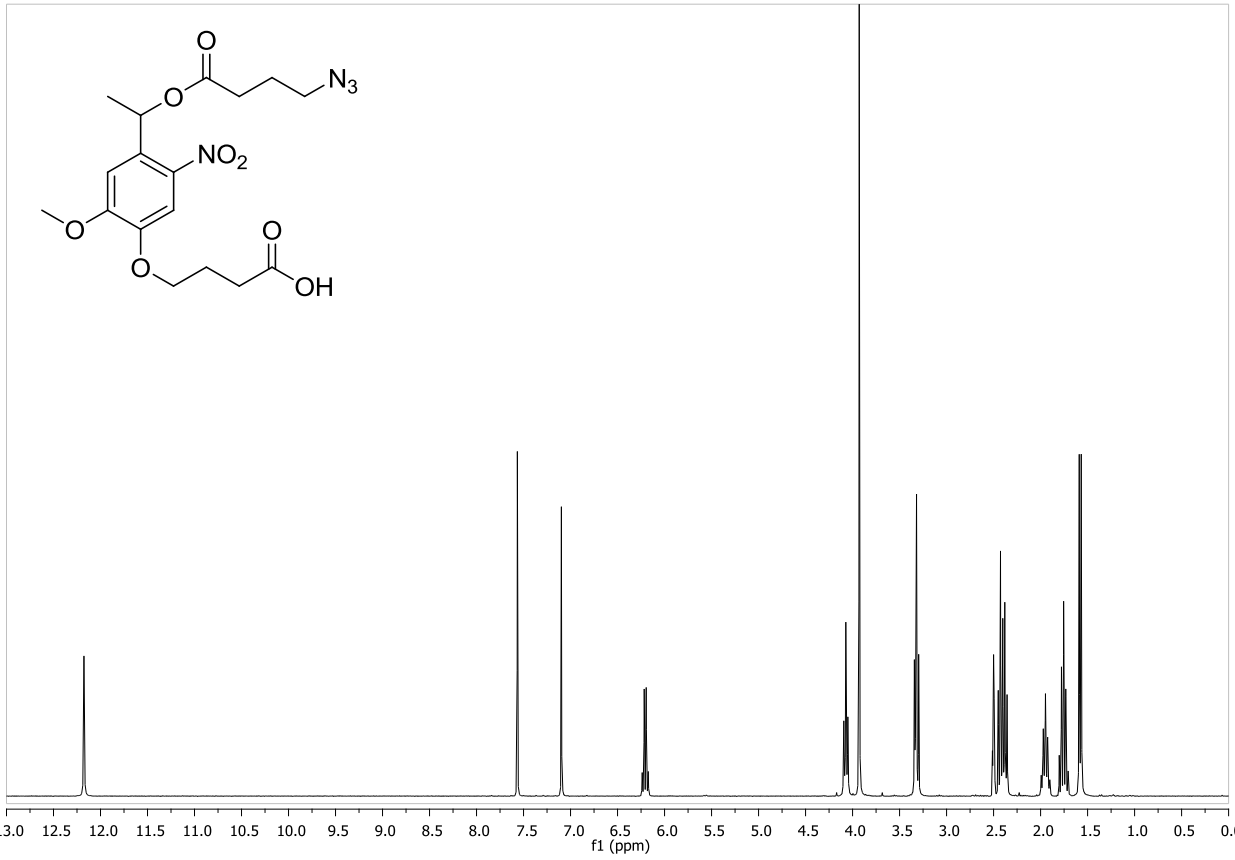


Figure S14 MALDI-TOF of Azide-RGK(alloc)GRK(PLazide)-NH_2

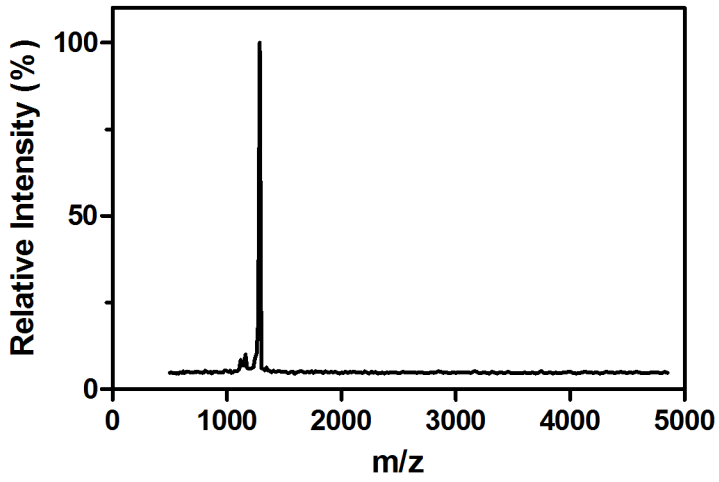
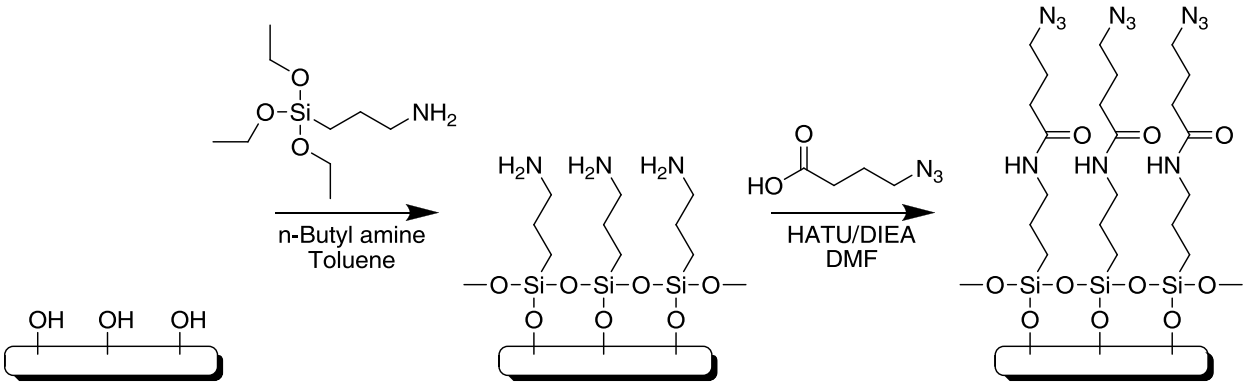


Figure S15 Synthesis of azide-functionalized glass slides



Glass slides (Fisher) were cleaned with piranha solution (50% sulfuric acid, 35% dH_2O, 15% H_2O_2) for 30 min at room temperature. The slides were dried after rinsing with water and acetone (3x) and then placed in a solution of (3-aminopropyl)-triethoxysilane (70 mM) and n-butyl amine (70 mM) in toluene for 90 min. The slides were cleaned with toluene, wiped dry, and baked at 80 °C overnight. The amine-functionalized slides were submerged in a solution containing 4-azidobutanoic acid (See Supplementary Fig. S13, 100 mM), HATU (100 mM), and DIEA (100 mM) in DMF. After 3 hours, the slides were rinsed with acetone and dried prior to use.

The amine-functionalization of this silanization procedure is derived from the work by Walba, *et al*[1].

1. Walba, D.M. et al. Self-assembled monolayers for liquid crystal alignment: simple preparation on glass using alkyltrialkoxysilanes. *Liq Cryst* **31**, 481-489 (2004).