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Visible Light Activation of Nucleophilic Thiol-X Addition via Thioether Bimane Photocleavage for Polymer Crosslinking

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KEYWORDS. Bioorthogonal, Michael addition, photo-crosslinking, visible light, nucleophilic thiol-X, hydrogel, polymer coupling.

ABSTRACT. On-demand photo-uncaging of reactive thiols have been employed in engineering biomaterial scaffolds for regulation of cellular activities. A drawback of the current photo-uncaging chemistry is the utilization of high energy UV light or 2-photon laser light, which may be harmful to cells and cause undesired side reactions within the biological environment. We introduce an effective approach for the caging of thiol using monobromobimane, which can be removed under irradiation of light at λ = 420 nm and in the presence of electrophiles, such as

acrylate, propiolate and maleimide, for trapping of the newly release thiol. This chemical approach can be used in visible light-induced polymer coupling and crosslinking for the preparation of cell-laden hydrogels.

Introduction

Photochemistry has emerged as a powerful tool for polymer modifications which are indispensable in advanced functional materials and biomedicine.[1-4] Light can endow spatial and temporal control in applications such as surface patterning, engineering 3D biomaterials structures, and on-demand drug release.[1] Photo-induced free radical polymerisation is perhaps the most popular chemical approach towards construction of biomaterial scaffolds with spatiotemporal regulation over the chemical and physical properties.[4] However, the generated free radicals may react with biological components such as cells and tissues,[5] causing irreversible damage. Although employment of photo-induced thiol-ene reaction has allowed for encapsulation of cells[6-8] and proteins[9] without affecting their bioactivity, photo-modulation of gel properties still requires the generation of reactive free radicals, which can have significant effect on radical-sensitive cells.[10]

To circumvent the generation of free radicals, photoremovable protecting groups (PPG) have been recently employed for photo-release of clickable functionalities in light-triggered polymer crosslinking.[2] For example, the photocleavage of *o*-nitrobenzyl (*o*-NB) derivatives was used to generate aldehyde,[11-13] ketone[14] or alkoxyamine[14, 15] for oxime or imine ligation in the construction of bioorthogonal hydrogels. The *o*-NB group has also been used in photo-uncaging of reactive thiol which can participate in subsequent Michael addition for modification of hydrogel microstructures.[16-18] Caging of the thiol group prevents its oxidation to disulfide under air exposure, thus increasing the storage of the thiol-containing polymers as well as the

efficiency of polymer ligation, via thiol-nucleophilic addition, prior to uncaging.[1] However, deprotection of o-NB thioether produces nitrosobenzaldehyde which can also react with the free thiols or amines within biological environments.

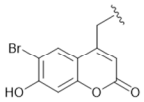
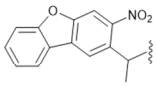
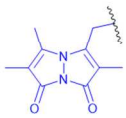
PPG		$\lambda_{uncaging}$	$\Phi_{uncaging}$	Literature	Applications in hydrogels engineering
	*o*-NB	325-405 nm	0.01	Ref. 14-16	Patterning and attachment of bioactive molecules (-) Nitrosoaldehyde-thiol side reactions
	Br-Cmr	365 nm	0.04	Ref. 17-19	Patterning and attachment of bioactive molecules (-) Photoisomerisation side reaction
	NDBF	365 nm or 740 nm (2-photon)	0.2	Ref. 22, 23	Patterning and attachment of bioactive molecules
	bimane	420 nm	0.02	This work	Bioorthogonal crosslinking/cell encapsulation

Figure 1. Comparison of PPGs used in photo-uncaging of thiols for engineering hydrogel structures.

The group of Shoichet utilised 6-bromo-7-hydroxycoumarin (Br-Cmr, **Figure 1**) to mask thiol groups within an agarose gel which, upon irradiation of either UV light or two-photon near infrared light, exposed free thiol.[19-21] Hence immobilisation of different growth factors in a spatial and temporal manner could be achieved, via thiol-maleimide addition, for guiding the differentiation of encapsulated stem cells. Similar to *o*-NB photo-uncaging, UV light irradiation of Br-Cmr group leads to photoisomerization side-product that can react with the newly released thiol, thus reducing the efficiency of the subsequent thiol-nucleophilic addition. To circumvent the photoisomerization of Br-Cmr, Shoichet and co-workers recently employed nitrodibenzofuran (NDBF) for concealment of active thiols within hyaluronic hydrogels.[22] Nevertheless, removal of the aforementioned PPGs can only by activated by UV light or two-

photon near-infrared light,[23] which may be damaging to cells and tissues at high dosage of light.[24-26]

For a PPG to be expedient in biomaterials engineering, it should fulfil several requirements[2] including: (i) negligible toxicity of the photoprotecting group and released by-products; (ii) high uncaging efficiency with minimal side reactions of the photo-released species under irradiation of wavelength ≥ 365 nm; and (iii) adequate solubility and stability in biological media. Ideally the photolabile precursor should also (iv) be easily synthesised and conjugated to the polymer structure. Taking all these prerequisites into consideration, we selected bimane chromophore as the PPG to mask the thiol group on polymer chains. Herein we report an efficient method for caging the thiol group using bromobimane. The photo-activated thiol can then be utilised in visible light-controlled polymer coupling, via nucleophilic thiol addition to various electrophiles including acrylate, propiolate and maleimide. In turn, we demonstrate the catalyst-free visible light-triggered crosslinking of poly(ethylene glycol) suitable for the fabrication of cell-laden hydrogels.

Experimental Section

General Considerations

Materials. Solvents (CH_2Cl_2, diethyl ether, petroleum ether, acetone, tetrahydrofurane, and methanol) were purchased from VWR in HPLC grade. 4-arm PEG10k-OH was purchased from Jenkem Tech, USA. 4-arm PEG10k-NH_2 was synthesised from 4-arm PEG10k-OH according to a previously published procedure with the degree of conversion from -OH to -NH_2 group of 96%.[27] Tetramethylrhodamine (TRITC) conjugated streptavidin was purchased from Thermo Fisher Scientific. All other chemicals were purchased from Sigma-Aldrich and used as received. All synthesized compounds were stored in a freezer at -20 °C and covered with aluminium foil.

Glassware for synthesis was first treated in a base bath (KOH in isopropanol 2 M) overnight, rinsed with water, submerged in an acid bath (HCl 1 M) for 2-4 h, rinsed with reverse osmosis water and dried in an oven at 120 °C for 6-12 h.

Chromatography. Thin-layer chromatography (TLC) was performed on silica gel 60 F254 alumina sheets (Merck) and visualized by UV light or potassium permanganate solution. Column chromatography was run on silica gel 60 (0.04-0.06 mm, 230-400 mesh ASTM, Merck).

NMR spectra were recorded on a Bruker Avance III 400 MHz or 600 MHz with a 5 mm broadband auto-tunable probe with Z-gradients at 293 K. Chemical shifts are reported as δ in parts per million (ppm) and referenced to the chemical shift of the residual solvent resonances ($CDCl_3$ δ = 7.26 ppm), couplings are shown as s: singlet, d: doublet, t: triplet, m: multiplet. Polymer samples were prepared at a concentration of 20 mg mL^{-1}. In most spectra traces of water appears as a broad singlet at around 1.5-2.5 ppm. NMR spectra were processed using MestReNova software.

Synthesis Procedures

Synthesis of 3-(bromomethyl)-2,5,6-trimethyl-1H,7H-pyrazolo[1,2-a]pyrazole-1,7-dione (monobromo bimane). A solution of hydrazine hydrate (6 mL, 0.16 mol) in ethanol (60 mL) was added dropwise to a solution of ethyl methylacetoacetate (17 mL, 0.12 mol) under stirring at ambient temperature. The solution was stirred for 2 h and concentrated in vacuo to give product as white solid (yield: 10.5 g, 78%).

The above product was suspended in chloroform (500 mL) and Cl_2 was bubbled through the solution via a glass pipette at a rate at which the solution was gently refluxed at ambient temperature. The white solid quickly dissolved into the solution and the mixture turned yellow

after 2 h. The solution was concentrated in vacuo to give yellow oil product which was used directly in the next step.

The above product was dissolved in CH_2Cl_2 (100 mL) and K_2CO_3 (17 g, 0.12 mol) was added. The solution was cooled on an ice bath and water (2 mL) was added slowly. The solution was then stirred at ambient temperature for 24 h, filter, and washed with saturated NH_4Cl solution. The organic phase was dried ($MgSO_4$) and concentrated in vacuo to give product as pale yellow solid which is mostly *syn*-bimane as indicated by ^{1}H NMR (total yield: 12 g, ca. 52%).

The above product (2 g, ca. 10.4 mmol) was dissolved in CH_2Cl_2 (20 mL) and cooled to 0 °C, a solution of Br_2 (0.5 mL, 9.72 mmol) in CH_2Cl_2 (40 mL) was added dropwise over 1 h. The solution was stirred at ambient temperature for 2 h and washed with water (100 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography running on ethyl acetate to give product as yellow solid (yield: 1.21 g, 45.9%). ^{1}H NMR (δ, ppm): 4.25 (s), 2.38 (s), 1.84 (s), 1.79 (s); ^{13}C NMR (δ, ppm): 6.8, 11.4, 17.5, 113, 115, 142, 144, 157, 158.

*Synthesis of 4-arm PEG-SH (**1**).* 4-arm PEG-OH (5 g, 0.5 mmol), mercaptopropionic acid (2.16 g, 20 mmol) and para-toluenesulfonic acid (10 mg, catalytic amount) were added to a solution of cyclohexane (100 mL) in a 250 mL round bottom flask. The solution was heated at 100 °C under Dean-Stark conditions for 16 h. The solution was cooled to ambient temperature and concentrated in vacuo. The residue was dissolved in dichloromethane (50 mL), washed with saturated $NaHCO_3$ solution (50 mL), water (50 mL), brine (50 mL), dried ($MgSO_4$), concentrated to ca. 5 mL and precipitated by dropwise addition in diethyl ether (200 mL). The polymer product was collected as white powder (yield: 4.6 g, 88.5%). The degree of end-group functionalization, calculated based on integration of NMR chemical shifts, was 99%. ^{1}H NMR

(δ, ppm): 4.24-4.26 (t, *J* = 4.8 Hz), 3.61 (broad s), 3.57 (s), 2.75-2.77 (q, *J* = 8.4, 7.3 Hz), 2.69 (t, = 7 Hz), Mn = 14300 g mol^{-1}, *Đ* = 1.13.

*Synthesis of 4-arm PEG-S-bimane (**2**).* 4-arm PEG-SH (1 g, 0.1 mmol) and monobromobimane (120 mg, 0.48 mmol) were dissolved in CH_2Cl_2, NEt_3 (48 mg, 0.48 mmol) was added and the solution was stirred at ambient temperature for 5 h. It was then precipitated into Et_2O to give product as pale yellow solid (yield: 0.96 g, ca. 95%). The degree of functionalization was 100%. ^{1}H NMR (δ, ppm): 4.25-4.27 (t, *J* = 4.8 Hz), 3.63 (broad s), 3.4 (s), 2.83-2.86 (t, *J* = 7.1 Hz), 2.67-2.68 (t, *J* = 7.1 Hz), 2.39 (s), 1.9 (s), 1.83 (s); M_n = 15100 g mol^{-1}, *Đ* = 1.21.

*Synthesis of 4-arm PEG-acrylate (**4a**).* 4-arm PEG-OH (1 g, 0.1 mmol) and NEt_3 (80 mg, 0.8 mmol) were dissolved in CH_2Cl_2 (20 mL) and the solution was cooled on an ice bath. Acryloyl chloride (72.4 mg, 0.8 mmol) in CH_2Cl_2 (5 mL) was added dropwise. The solution was allowed to warm to ambient temperature and stirred for 6 h after that it was washed with water (50 mL x2), brine (50 mL), dried (MgSO4), and concentrated in vacuo to ca. 2 mL and precipitated by dropwise addition in diethyl ether (100 mL) to give product as white solid (yield: 0.88 g, ca. 88%). The degree of end-group functionalization was 99%. ^{1}H NMR (δ, ppm): 6.40-6.44 (dd, J = 1.4, 15.9), 6.11-6.18 (dd, J = 10.5 Hz, 6.6 Hz), 5.82-5.85 (dd, J = 1.4 Hz, 8.8 Hz), 4.30-4.31 (t, J = 4.8 Hz), 3.72 (broad s), 3.66 (s). M_n = 13300 g mol^{-1}, Đ = 1.07.

Synthesis of 4-arm PEG-propiolate. 4-arm PEG-OH (2 g, 0.2 mmol), propiolic acid (0.14 g, 2 mmol), p-toluene sulfonic acid (10 mg, catalytic amount) were added to a solution of cyclohexane (100 mL) in a 250 mL round bottom flask. The solution was heated at 100 °C under Dean-Stark conditions for 16 h. The solution was cooled to ambient temperature and concentrated in vacuo. The residue was dissolved in dichloromethane (50 mL), washed with saturated $NaHCO_3$ solution (50 mL), water (50 mL), brine (50 mL), dried ($MgSO_4$), concentrated

to ca. 2 mL and precipitated by dropwise addition in diethyl ether (100 mL). The polymer product was collected as white powder (yield: 1.7 g, ca. 85%). The degree of end-group functionalization was 99%. ^{1}H NMR (δ, ppm): 4.34-4.36 (t, J = 4.8 Hz), 3.66 (bs), 3.65 (s), 3.0 (s). M_n = 13900 g mol^{-1}, Đ = 1.09.

Synthesis of 2,5-dioxopyrrolidin-1-yl 3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanoate (N-hydroxysuccinimide maleimide). Glycine (1.5 g, 20 mmol) and maleic anhydride (2.35 g, 2 mmol) were dissolved in acetic acid (50 mL) and the solution was stirred at 130 °C for 5 h. The solution was then allowed to cool to ambient temperature and poured into water (200 mL), extracted with EtOAC (100 mL x 3), dried ($MgSO_4$), and concentrated in vacuo to give product as white crystal.

The above product was redissolved in CH_2Cl_2 (50 mL) and to the solution was added EDC.HCl (3.82 g, 20 mmol) and N-hydroxysuccinimide (2.26 g, 20 mmol). The solution was stirred at ambient temperature for 2 h, washed with water (50 mL), brine (50 mL), dried ($MgSO_4$), concentrated and purified by column chromatography eluting with EtOAC/CH_2Cl_2 (v/v = 1/4) to give product as white crystal (total yield: 4.2 g, 79%). ^{1}H NMR (δ, ppm): 6.73 (s), 3.93 (t, J = 7.0 Hz), 3.03 (t, J = 7.0 Hz), 2.82 (bs).

Synthesis of 4-arm PEG-maleimide. N-hydroxysuccinimide maleimide (109 mg, 0.41 mmol) and 4-arm PEG10k-NH_2 (1 g, 0.1 mmol) were dissolved in CH_2Cl_2 (10 mL) and the solution was stirred at ambient temperature for 4 h. The mixture was then precipitated into Et_2O (200 mL) to give product as white powder (yield: 1 g, ca. 92%). The degree of end-group functionalization was 97%. ^{1}H NMR (δ, ppm): 6.84 (bs), 6.71 (s), 3.84-3.87 (t, J = 7.2 Hz), 3.66 (bs), 3.42 (bs), 2.52-2.55 (t, J = 7.2 Hz). M_n = 14400 g mol^{-1}, Đ = 1.13.

Characterization Methods

Light irradiation is undertaken using a Mightex's WheeLED wavelength-switchable LED for λ = 420 nm (built-in filter). The intensity of light irradiance on the gel sample was tuned to the desired intensity using a RM-12 radiometer (Opsytec) with a sensor VISBG 400-570 nm.

Ellman's test: The amount of free thiol released under visible light irradiation was determined using the Ellman's test. In a typical procedure, an aqueous solution of 4-arm PEG-Sbimane (1.5 mL, 0.05 mM, ca. 0.2 mM of the –Sbimane endgroup, Tris buffered pH 7.4) in a quartz cuvette equipped with a stirring bar was first corrected as background on the Cary60. To this solution was added a solution of 5,5'-dithiobis-(2-nitrobenzoic acid) (0.2 mM in Tris buffered pH 7.4) and the mixture was irradiated with blue light (420 nm, 20 mW cm^{-2}) under stirring for predetermined periods of time. The solution was allowed to react with stirring in the dark for 10 min and the absorbance at 412 nm was recorded. L-cysteine solutions (0.025-0.2 mM) were used as standards for calculation of the amount of thiol release. Each measurement was done in triplicate.

Quantum efficiency. Data of the progress of Ellman's assay were plotted in Sigmaplot 12.5 and fitted via nonlinear regression analysis to a first-order process. The quantum efficiency (Φ_u) was calculated using the equation $\Phi_u = (I\sigma t_{70\%})$, where I is the irradiation intensity in Einstein cm^{-2} s^{-1}, σ is the decadic extinction coefficient ($10^3 \times \varepsilon$, molar extinction coefficient at 420 nm) in cm^2 mol^{-1}, and $t_{70\%}$ is the irradiation time in seconds for 70% conversion to the product.[23] The light intensity was re-measured using potassium ferrioxalate actinometry.

Size exclusion chromatography (SEC) was performed using a Shimadzu modular system comprised of a SIL-20AD automatic injector, an RID-10A differential refractive-index detector and a 50 × 7.8 mm guard column followed by three KF-805L columns (300 × 8 mm, bead size: 10 μm, pore size maximum: 5000 Å). N,N-Dimethylacetamide (HPLC grade, 0.03% w/v LiBr)

at 50 °C was used for the analysis with a flow rate of 1 mL min^{-1}. Reaction time course samples dissolved in DMAc were filtered through 0.45 μm PTFE filters before injection. The SEC calibration was performed with narrow-polydispersity polystyrene standards ranging from 500 to 2 × 106 g mol^{-1}.

Fluorescence spectrum was obtained using a Shimadzu RF-5301 PC fluorescence spectrophotometer. 2.5 mg of (reaction time course) sample was diluted in 3 mL of 1:2 acetonitrile/H_2O and then further diluted 1 in 10 (200 μL added to 1.8 mL of H_2O). Fluorescence spectra were run using excitation wavelength 390 nm and emission scanned between 400 and 800 nm.

Rheological data were recorded using an Anton Paar Physica rheometer with a plate-plate configuration. The lower plate is made of quartz and the upper plate is made of stainless steel with a diameter of 15 mm. A liquid light guild, which was connected to the light source was equipped below the quartz plate. In a typical experiment, a polymer solution (50 μL, 10 wt%) of 4-arm PEG-SH/PEG-Sbimane and a solution of 4-arm PEG-Acr/Pro/Mal (50 μL, 10 wt%) were mixed on a vortex mixer for 5 seconds. This solution was placed on the lower plate and the upper plate was lowered to a measurement gap of 0.2 mm. A layer of paraffin oil was applied on the edge of the stainless steel plate to prevent dehydration of hydrogel and the test was started by applying a 1% strain with the frequency of 0.1 Hz on the sample. In all rheological tests, an induction period of 20-200 s was observed after light illumination and before a sharp increase in the storage modulus.

Hydrogel patterning. A solution (300 μ*L*) of 4-arm PEG-Acr (c = 8 mM) and 4-arm PEG-Sbimane (c = 10 mM) was casted into a circular Teflon mould with a diameter of 1 cm. The solution was then subjected to light irradiation (λ = 420 nm, I = 20 mW cm^{-2}) for 10 min, after

which a yellow solid gel was formed. The gel was then immersed in an aqueous solution of biotin-maleimide (c = 0.1 mM, Sigma Aldrich) for 1 h. A photomask was then placed on the surface of the gel and the light irradiation was applied on the materials for 20 min. The hydrogel was then placed in an aqueous solution of TRITC-streptavidin (c = 0.1 mg mL^{-1}) for 1 h. The material was then washed with water/ethanol solution (v/v = 8/2) was then subjected to imaging under a Nikon fluorescent microscope.

Human mesenchymal stem cell culture. hMSCs (Bone marrow-derived) were cultured in low glucose-DMEM (1 g L^{-1} $_D$-glucose and 110 mg L^{-1} sodium pyruvate) with 100 μg mL^{-1} penicillin-streptomycin and 10% (v/v) fetal bovine serum (FBS) supplements and maintained at 37 °C and 5% CO_2. Passage 6 cells were used in this work.

hBMSC encapsulation and viability test. hMSCs were harvested by TrypLE™ Express treatment, spun down and resuspended in three different electrophile-containing crosslinkers stock solutions (4-arm PEG-Arc, 4-arm PEG-Pro and 4-arm PEG-Mal). The cell/crosslinker mixtures were then mixed thoroughly with 4-arm PEG-Sbimane to achieve the final cell density of 2.5 million cells mL^{-1}. Triplicate samples (25 μL/each) per condition were prepared in a culture dish and subsequently exposed to the 420 nm light (I = 20 mW cm^{-2}) for 10 minutes.

The crosslinked cell-laden hydrogel samples were rinsed twice with culture media and maintained at 37 °C and 5% CO_2. To test cell viability, live/dead staining was performed after 24 hrs encapsulation. Gels were washed with phosphate buffer saline (PBS) solution and incubated with Live/Dead staining kit (Life Technologies, USA) for 30 minutes at room temperature. The samples were then rinsed thrice with PBS and imaged using a Nikon fluorescent microscope.

Results and Discussion

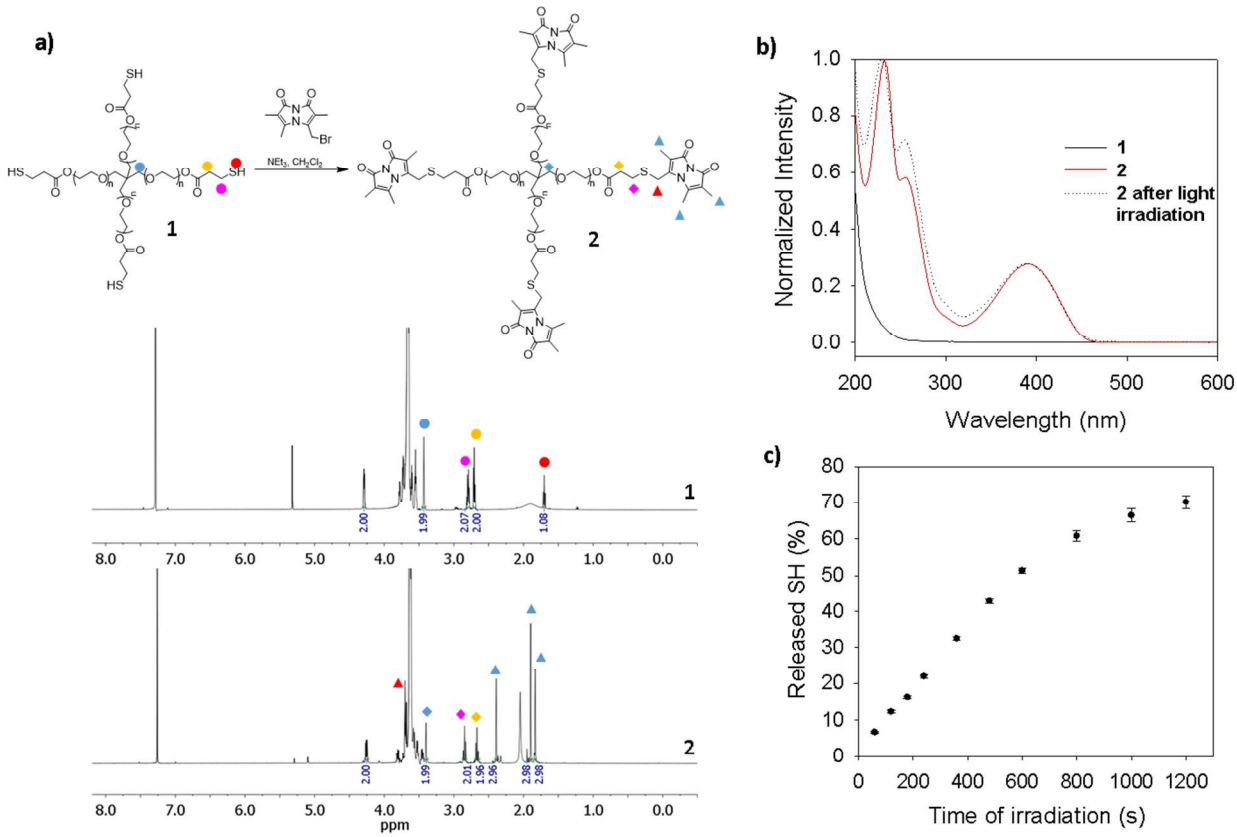


Figure 2. a) Scheme of the caging thiol end-groups using monobromobimane, and ^{1}H NMR spectra of the polymers ($CDCl_3$, 600 MHz); b) UV-vis spectra of the 4-arm PEG-SH in aqueous solution (0.05 mM) before and after caging with bimane, and after irradiation at λ = 420 nm for 30 min; and c) graph showing the trapping of bimane-protected thiol, determined by Ellman's test, followed by irradiation at different time periods (error bars represent standard deviations of 3 repeats).

The bimane structure is highly compact and this group has good solubility in aqueous solvent which is useful for preparation of water soluble conjugated polymers for biological studies.[28] Bromobimane has high reactivity towards free thiol and has been used for fluorescent tagging of proteins.[28-30] The thio-bimane ether molecules have been shown to be highly stable and non-toxic to cells.[30] Here we employ this reagent to protect thiol end-groups of a 4-arm PEG-SH (**1**) via

thiol-bromo reaction (**Figure 2a**). Although bromobimane is commercially available, this reagent is expensive which prompts us to prepare bromobimane from reported procedures.[31, 32] In our hands, the synthesis of bromobimane can be streamlined to a continuous 3-step process with only one column chromatography purification, and an overall yield of 17.9% in gram scale (See Supporting Information for details). This yield is lower than the yield previously reported for monobromobimane synthesis, which was ca. 24%, and the lower yield may be due to some intermediates not purified in our synthesis procedure.[28,29] The 4-arm PEG-SH can be easily prepared by Fischer's esterification of 4-arm PEG-OH using *para*-toluenesulfonic acid as the catalyst, and complete conversion of the –OH to –SH group was obtained.

Treatment of the 4-arm PEG-SH with bromobimane in dichloromethane and in the presence of trimethylamine led to complete caging of the –SH group as indicated by ^{1}H NMR analysis (**Figure 2a**). The resultant polymer (**2**) has absorption and emission maxima at 388 nm (molar extinction coefficient ε_{388} = 6.8 x 10^3 L mol^{-1} cm^{-1}) and 472 nm respectively (Stoke's shift of 84 nm) in aqueous solution (**Figure 2b** and **Figure S10**). Based on the UV-vis spectrum we selected a 420 nm LED light source, with an irradiance tuned at 20 mW cm^{-2}, to investigate the photocleavage of the thio-bimane group. Under stirring in a Tris-buffered (pH 7.4) solution containing Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid), we observed circa 70% of the Sbimane was photo-reacted with Ellman's reagent (**Figure 2c**) after 20 minutes of light irradiation. The quantum yield of this photoreaction was calculated to be 0.02. The incomplete release of the thiol may be due to the competing absorbance of the bimane-hydroxyl product and competing recombination reaction as shown below.

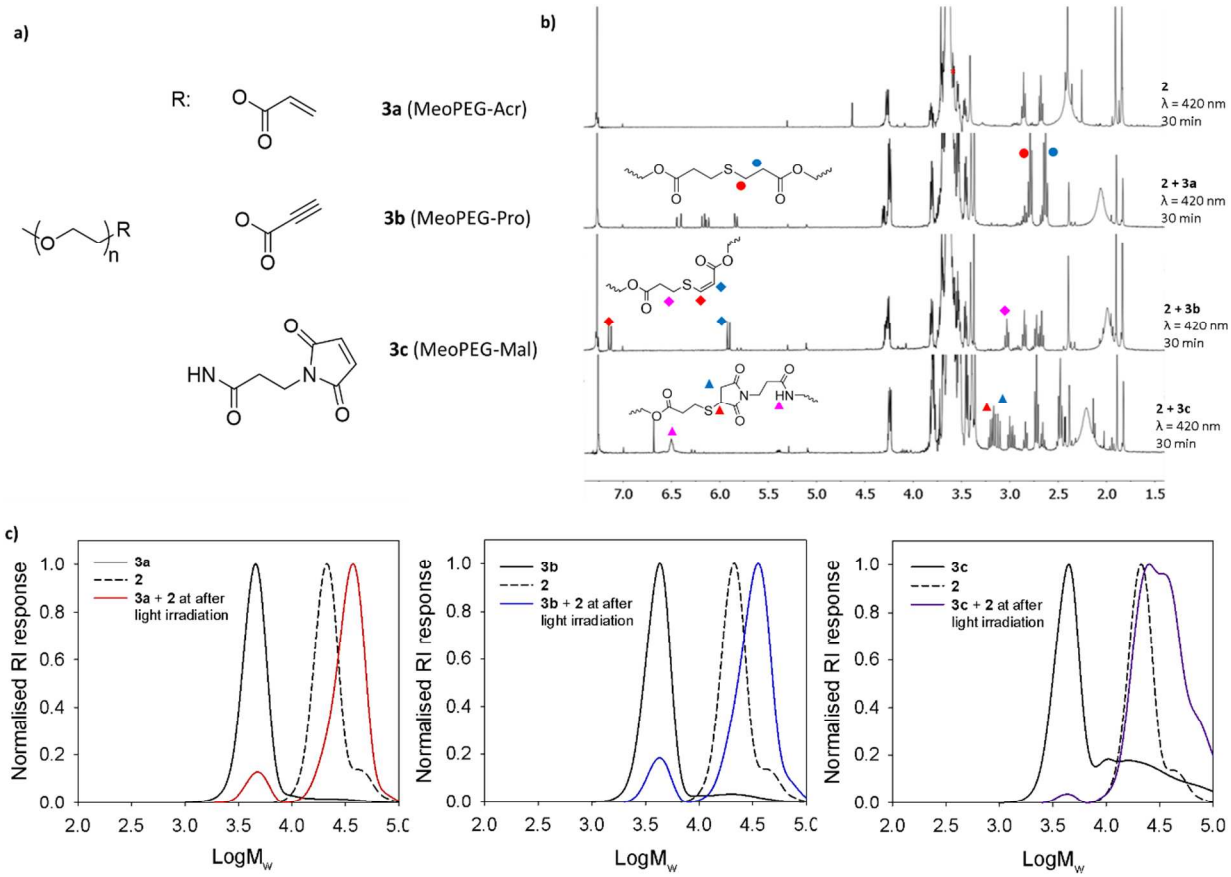
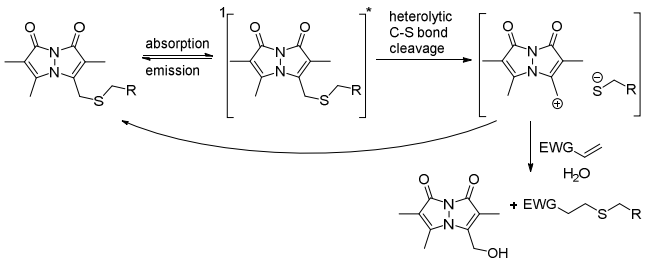


Figure 3. a) Structures of the PEG with electrophiles end-groups for thiol-addition; **b)** ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of the polymers after light treatment showing chemical shifts from the thiol addition reactions; and **c)** SEC traces of **2** before and after irradiation at 420 nm with and without the electrophiles, indicating polymer ligation via thiol-nucleophilic addition.

We next analyzed **2** after photo-uncaging by extracting the polymer from aqueous solution using dichloromethane followed by precipitation in diethyl ether. To our surprise, we did not observe free thiol, as indicated by the Ellman's test, when examining ^{1}H NMR spectrum of the 4-arm PEG-Sbimane (**2**) after light irradiation in the absence of the Ellman's reagent (**Figure 3**). This indicates that no reaction occurred when irradiating aqueous solution of PEG-Sbiman. On the other hand, when solution (Tris-buffered, pH 7.4) of **2** was irradiated in the presence of linear PEGs having electrophilic end-groups (**Figure 3a**) such as acrylate (Acr), propiolate (Pro) and

maleimide (Mal), we observed formation of polymer-polymer coupling product as indicated by the peaks at higher molecular weight in SEC traces (**Figure 3c**). The presence of the thiol-nucleophilic addition adducts is also evident in the ^{1}H NMR spectra (**Figure 3b**) of the polymers extracted from the reacting solution. In the reaction of photo-reactive thiol with propiolate, the chemical shifts of the major alkenyl protons display a vicinal coupling constant ($^{3}J_{HH}$) of 10 Hz, which is characteristic of the *cis*-isomer. This is in agreement with previous reports[33, 34] on nucleophilic thiol-propiolate addition in PBS, which typically produces *cis*-alkenyl thioether. Integration of the corresponding chemical shifts allows us to calculate the efficiency of the free thiol reacting with the electrophilic counterparts. Specifically, the acrylate group gave the highest trapping efficiency of the photo-released thiol with 72% conversion observed, whereas only 63% and 51% conversions were calculated for propiolate and maleimide respectively. The lower conversion of Sbimane in the reaction with maleimide may be due to photo-cycloaddition side reactions of the maleimide group.[35] The undefined nature of the Sbimane-maleimide photoreaction is further shown by the bimodal characteristic of the SEC trace of the polymer product.

Scheme 1. Suggested mechanism for the photo-uncaging of thiol bimane in the presence of an electrophile.



Based on our observation of ^{1}H NMR data and previous work on photolysis quenching of ester bimane by Sigh and co-workers,[36] we propose a mechanism for the photo-uncaging of thioether bimane as outlined in **Scheme 1**. After photon absorption, the bimane thioether is converted to the singlet excited state S_1, which then can fluoresce or undergo heterolytic C-S bond cleavage to form a zwitterion intermediate. The zwitterion intermediate can recombine back to the ground state thioether bimane or react with electrophiles such as electron-deficient alkene/alkyne in water to form the respective thioether and hydroxyl bimane by-product. The competing recombination reaction, as opposed to solvent trapping and hydrolysis in aqueous solvent, may contribute to the stability of **2** in solution under light irradiation and in the absence of electrophiles. Furthermore, the incomplete photocleavage in the presence of electrophiles, as seen in the Ellman's test and nucleophilic addition with acrylate, propiolate and maleimide, is probably due to the recombination of the zwitterion intermediate. Since the hydroxyl bimane by-product has a maximum absorbance of 325 nm in water, a blue shift from the absorbance of the Sbimane group,[37] it would be expected the competing absorbance of this molecule is minimal. Meanwhile, we observed excellent stability of **2** in phosphate buffer saline solution under ambient storage condition and under light exposure for 2 weeks, i.e. no changes observed in ^{1}H NMR spectrum. This high stability of the protected thiol under light and in the absence of the electrophiles can be highly advantageous for storage of materials and retaining the photoreactivity of protected thiols before photo-induced conjugation.

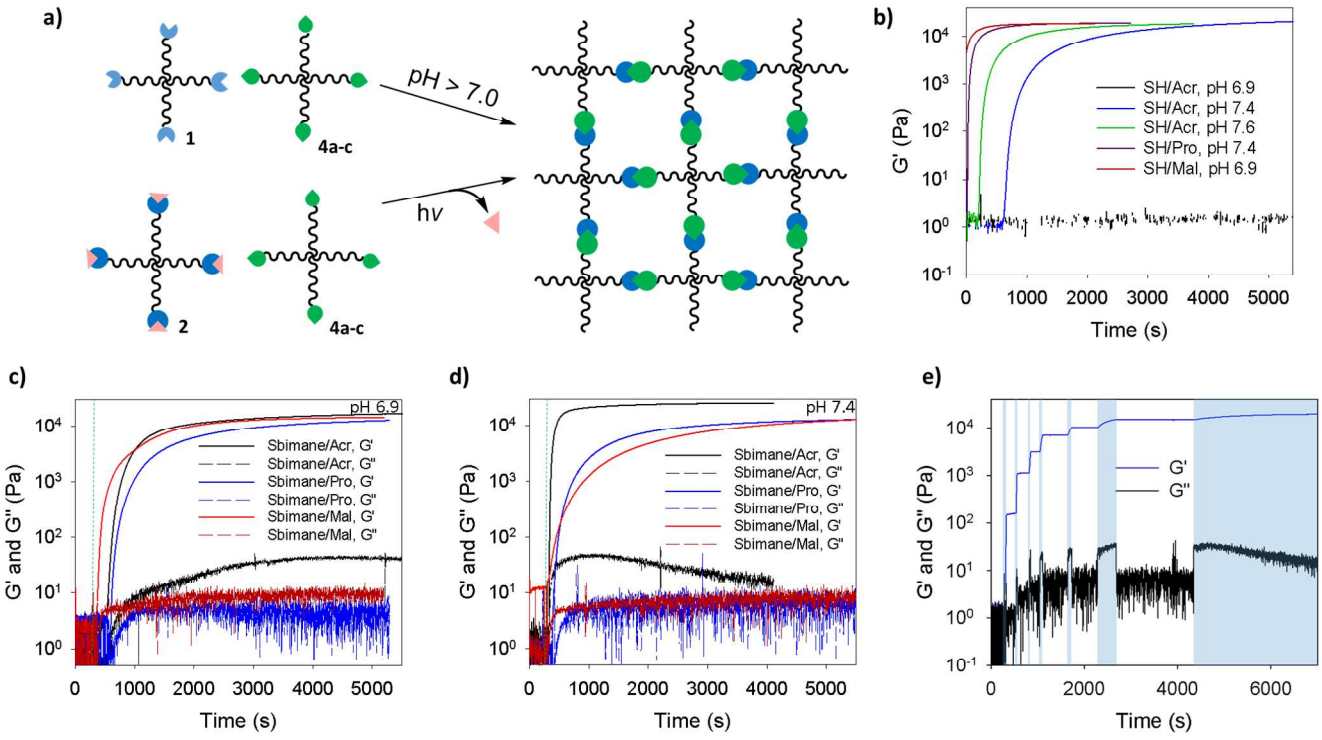


Figure 4. a) Scheme of polymer crosslinking via spontaneous and light-triggered thiol-nucleophilic additions; **b)** Gelation profiles of spontaneous thiol-nucleophilic addition followed by the increase in storage moduli of the polymer solutions (c = 10 mM) at different pH; Gelation profiles of thiol-nucleophilic addition via light-triggered release of thiol under irradiation of λ = 420 nm and I = 20 mW cm^{-2} and at **c)** pH 6.9 and **d)** pH 7.4, dashed green lines indicate when the light was switched on; and **e)** temporal control of the gelation process for Sbimane/Acr gel in PBS pH 7.4 by blue light (420 nm), highlighted areas indicate when the light was turned on.

Having proof that the photo-uncaging of thioether bimane could occur effectively in the presence of a nucleophile trap, we next prepared 4-arm PEG linkers containing similar electrophiles (**4a-c**) and assessed the polymer crosslinking under visible light irradiation using rheological tests. For comparison, we first studied the gelation of 4-arm PEG-SH with 4-arm

PEG linkers in phosphate buffer saline (**Figure 4a**). As seen in **Figure 4b**, the gelation kinetics, followed by the increase in the storage modulus of the mixture of the investigated crosslinkers, is highly dependent on the nature of the electrophile and pH. For acrylate end-group, a pH ≥ 7.4 is required for efficient crosslinking with complete gelation occurring after 40-60 min of mixing the crosslinker and 4-arm PEG-SH. Gelation was faster for the propiolate end-group at similar pH, with complete gelation in less than 10 min at pH 7.4. The SH/Pro crosslinking could also proceed effectively at slightly basic pH 7.1 (**Figure S11**). The maleimide group is highly reactive towards free thiol, as gels were observed to form almost instantaneously upon mixing PBS solutions (pH > 7.0) of 4-arm PEG-SH and 4-arm PEG-Mal. We were only able to obtain part of the gelation profile for SH/Mal crosslinking on the rheometer using distilled water (pH 6.9) as the medium.

Replacing 4-arm PEG-SH (**1**) with 4-arm PEG-Sbimane (**2**) allows for gelation to occur only when the polymer solutions were subjected to irradiation at λ = 420 nm and I = 20 mW cm^{-2} (**Figure 4c**). Interestingly, crosslinking could occur for 4-arm PEG-Acr in solution with pH 6.9, confirming that the released thiol is in anionic form. The gelation, featured by the cross-over between G' and G", generally occurred after 1-2 min of irradiation for both Sbimane/Acr and Sbimane/Pro solutions, whereas gelation happened more rapidly for Sbimane/Mal solution due to the higher reactivity of maleimide towards nucleophiles. In our [4+4] hydrogel system with equivalent molar ratio of the reacting groups, a 33% conversion of the functional groups is required for gelation to occur according to Flory Stockmayer's theory.[38, 39] In the absence of electrophile-containing linkers, no gelation was observed for 4-arm PEG-Sbimane under light irradiation (**Figure S12a**). Addition of hydrogen peroxide, which is known to induce thiol

oxidation, to solution of **2** resulted in a slight increase in the storage modulus after ca. 30 min of irradiation (**Figure S12b**). This shows that oxidation during light irradiation is not significant.

The gelation onset for Sbimane/Acr and SBimane/Pro occurred more rapidly when pH of the solution was raised to 7.4 (**Figure 4d**) and the effect of basic pH on the gelation kinetics is more profound for the Sbimane/Acr mixture, with complete gelation, featured by the G' value reaching a plateau, happened after ca. 20 min of blue light irradiation. This fast gelation indicates a faster trapping of the photo-reactive thiol compared to the observed results from Ellman's test, and this may be due to the higher reactivity of the acrylate compared to 5,5'-dithiobis-(2-nitrobenzoic acid) used in Ellma's assay. In comparison, slower gelation kinetics was observer for Sbimane/Pro and Sbimane/Mal mixtures, which is consistent to the lower trapping efficiency of both the Pro and Mal groups compared to the Acr group in reactions of 4-arm PEG-SH with linear MeO-PEGs. To account for the lower conversion of Pro and Mal in the reaction with photo-reactive thiols, we irradiated **4a**, **4b**, and **4c** separately in PBS pH 7.4 and in the absence of **2** for 1 h. ^{1}H NMR analysis of the polymers after light treatment shows that the Acr group remained intact whilst some side reactions occurred in both **4b** and **4c** solutions, resulting in 38% and 15% reduction of the Pro and Mal groups respectively (**Figure S6 and S9**). The loss of the alkyne in **4b** structure is accompanied by the loss of the ester group, indicating hydrolysis of the propiolate, whereas the loss of the maleimide in **4c** may be due to both and photolysis and free-radical dimerization.[35]

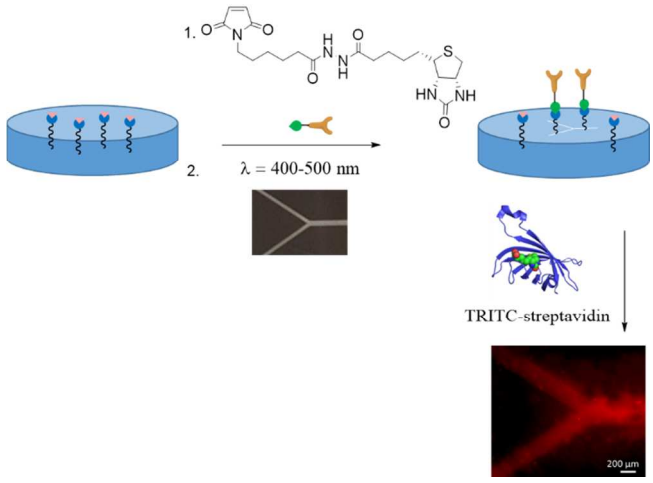


Figure 5. Photo-patterning of hydrogel containing Sbimane group with biotin-maleimide under irradiation of visible light, and subsequent reaction with TRITC-streptavidin. Image showing red fluorescently labelled streptavidin in the patterned region.

A distinct advantage of the photocrosslinking over spontaneous mixing-induced crosslinking in hydrogel preparation is the ability to fine-tune the gel mechanical properties in a temporal and spatial fashion. To demonstrate the temporal control over gel properties, we undertook a rheological experiment in which the storage and loss moduli of the Sbimane/Acr solution pH 6.9 were monitored while blue light was switched on and off continuously. As seen in **Figure 4e**, the polymerization was totally halted when the light was turned off, and resumed only when the light was on, demonstrating the defined controlled over photo-uncaging and trapping process. To demonstrate the spatial control over conjugation process, we first prepared a hydrogel containing unreated Sbimane groups within the gel network. Upon photolysis under visible light irradiation and in the presence of biotin-maleimide the Sbimane group reacted with maleimide in the irradiated regions (**Figure 5**). The immobilization of the biotin in the patterned area was confirmed by the specific reaction of biotin with TRITC-streptavidin, which can then be visualized under a fluorescence microscope.

Table 1. Physical properties of hydrogels formed by spontaneous and photo-triggered thiol-nucleophilic addition.

Hydrogels [a]	G'$_{as\text{-}prepared}$ (kPa)	G'$_{swollen}$ (kPa)	EWC[b] (%)	Gel fraction[c]
SH/Acr[d]	22.9 ± 1.7	13.1 ± 1.1	94.4 ± 1.1	91 ± 2
SH/Pro[e]	18.5 ± 1.2	8.7 ± 0.8	94.6 ± 1.3	87 ± 3
SH/Mal[f]	19.8 ± 1.9	9.5 ± 0.9	94.7 ± 1.2	89 ± 2
Sbimane/Acr[e]	20.8 ± 2.1	12.3 ± 1.2	96.1 ± 1.3	88 ± 3
Sbimane/Pro[e]	13.6 ± 2.7	5.4 ± 1.3	96.8 ± 1.4	71 ± 4
Sbimane/Mal[e]	14.1 ± 2.6	6.7 ± 1.3	96.3 ± 1.4	72 ± 4

[a]: hydrogels were prepared from 100 μL solution of polymer with a total polymer concentration of 10 mM (each reacting polymer solution had the concentration of 5 mM) and a resultant thickness of 1 mm, reported values are the averages of 3 samples and errors represent standard deviations; [b]: Equilibrium water content (EWC) was calculated from the following equation: $(w_{swollen}\text{-}w_{dry})$x100%/$w_{swollen}$ in which $w_{swollen}$ is the weight of fully swollen gel and w_{dry} is the weight of dried gel; [c]: gel fraction was calculated from the ratio of dry gel weight over weight of polymer before gelation; [d]: pH 7.6; [e]: pH 7.1; [f]: pH 6.9; [e]: pH 7.4.

We further characterised physical properties of the hydrogels prepared by both spontaneous polymerization and photo-polymerization, and the data are presented in **Table 1**. All photo-polymerized gels were prepared from solutions with a thickness of 1 mm to minimise light attenuation in photocrosslinking, and the irradiation time for photocrosslinking was 30 min at I = 20 mW cm^{-2}. The molar absorptivity of the polymer solutions were found to be 1.9 x 10^3 L mol^{-1} cm^{-1} and the resultant gels are transparent. The expected bimane hydroxyl molecule In general, the as-prepared hydrogels, with an initial water content of 90.9%, has robust mechanical strength with the storage moduli in the range of 14-23 kPa. These hydrogels were highly water absorbent with the equilibrium water contents varying from 94-96%. The G' values were also

observed to decrease as the gels were swollen in water.[40, 41] Compared to spontaneously-formed hydrogels, photocrosslinked gels have lower crosslinking efficiency, as shown by the lower storage moduli and gel fraction, indicating the photo-induced polymerized network is less defined than the spontaneously formed network. This may be due to several factors including: light attenuation, competing recombination of the ion-pair intermediate (**Scheme 1**) and light absorption of the hydroxyl bimane by-product. Nevertheless, light-induced crosslinked gels displayed good mechanical strength, even in their fully swollen state with the storage modulus values in the range of 5-12 kPa. The physical properties of photocrosslinked gels may be tuned within this range by altering the water content of the pre-gel solutions, molar stoichiometry of the reacting groups, or time of irradiation.[15, 41-44]

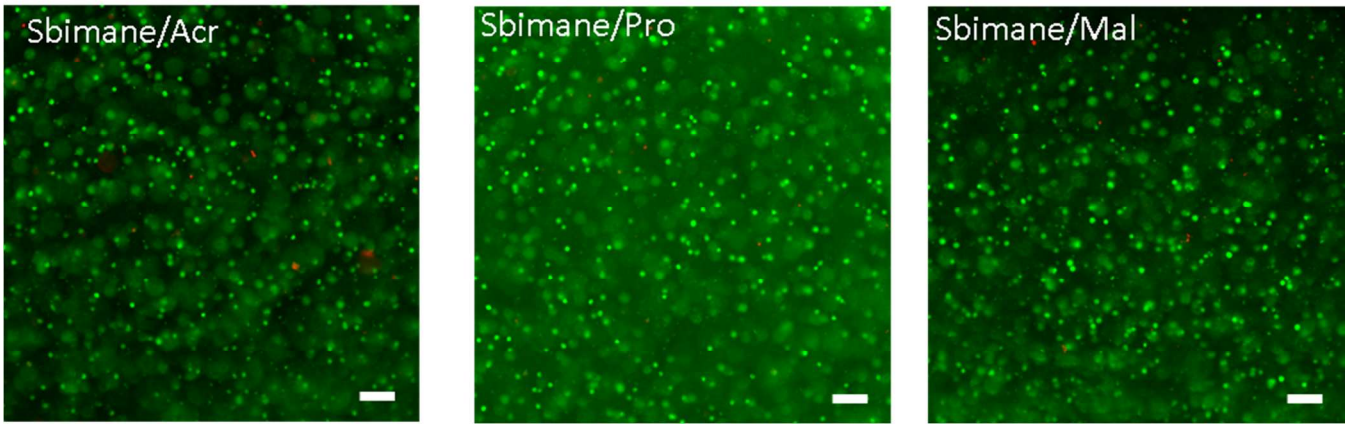


Figure 6. Live-dead staining of hMSCs 24-h post-encapsulation (live cell = green, dead cell = red, scale bar = 100 μm).

To assess the potential utility of our photo-uncaging/thiol-nucleophilic conjugation in biological studies, we carried out polymer crosslinking using all three electrophile-containing crosslinkers in the presence of hMSCs. The gelation condition was set at c = 10 mM and

irradiation at λ = 420 nm, I = 20 mW cm^{-2} for 10 min, which produced gels having thickness of 2-3 mm and moduli in the range of 2-4 kPa. Rheological analysis of the gelation process using cell culture media as the solvent showed similar kinetics as PBS pH 7.4, i.e. rapid gelation upon light irradiation (Figure S13). Live/dead staining of the encapsulated cells after 24 h post-gelation shows high percentage of live cells (>90% cell viability, **Figure 6**), which suggests the photocrosslinking process and hydroxyl bimane by-product are both non-toxic to living cells. In our cell encapsulation study, PEG-only hydrogel was used which limits the culture duration because PEG does not contain cell binding sequence. Therefore the viability of the cells decreased with increasing culture time.[43] For tissue engineering applications, hydrogels prepared from naturally derived polymers such as hyaluronic acid[1] and gelatin may be used to support cells growth.[7, 12, 43] These polymers can also be modified to attach the Sbimane group for spatial-tuning of gel stiffness and/or temporal-controlled activation of biomolecules.

Conclusions

In conclusion, we present an efficient approach for caging of the thiol group using bromobimane. Photo-uncaging of the thioether bimane under irradiation of blue light, coupled with trapping of the anionic thiols by electrophiles can proceed rapidly and efficiently in water and without the requirement of catalyst, providing a means for construction of photo-crosslinked gels with good mechanical properties. Importantly, the bimane group fulfils all requirements for a photocleavable caging group suitable for biology related studies. These requirements, as demonstrated in our work, include good water solubility, high stability in biological media and minimal toxicity to cells. The nucleophiles, including propiolate and maleimide, employed in this work were found to partially degrade under the light treatment conditions, nevertheless we expect that more stable electrophiles without the ester group such as vinylsulfone will

circumvent the stability issues encountered in the propiolate and maleimide groups. We believe the caging and photouncaging-coupling process introduced in this work can be effectively employed in a range of applications including molecular biology and spatiotemporal control of biomaterials properties to direct cell-materials interaction in regenerative medicine.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge.

NMR spectra of the synthesized compounds, additional UV-vis and fluorescent spectra, and additional rheological data (file type, PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

PEG, poly(ethylene glycol); *o*-NB, *ortho*-nitrobenzyl; NDBF, nitrodibenzofuran ; PPG, photoremovable protecting group; Cmr, coumarin; TLC, thin-layer chromatography; SEC, size exclusion chromatography; Acr, acrylate; Pro, propiolate; Mal, maleimide; PBS, phosphate buffer saline.

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