Supporting Information (SI)

Photoactive Methylene Blue-Functionalized Polymer for Antimicrobial Activation under Red Light

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EXPERIMENTAL SECTION

Materials

Methylene blue hydrate (Sigma-Aldrich, ≥ 95%), triphosgene (Sigma-Aldrich, 98%), hydrogen chloride solution (Sigma-Aldrich, 4 M in 1,4-dioxane), *N*-[2-(Boc-amino)ethyl]acrylamide (Accela ChemBio, ≥95%), *N*-hydroxyethyl acrylamide (Sigma-Aldrich, 97%), *N*-butylacrylamide (Ambeed, 98%), 2-(butylthiocarbonothioylthio)propanoic acid (Boron Molecular, 95%), 2,2'-azobis(2-methylpropionitrile) (AIBN) solution (Sigma-Aldrich, 12 wt% in acetone), 9,10-dimethylanthracene (Sigma-Aldrich, 99%), nitrotetrazolium blue chloride (Sigma-Aldrich, ≥ 90%), bovine serum albumin (Sigma-Aldrich, ≥ 96%), and propidium iodide (Sigma-Aldrich, ≥ 94%) were used as received. Sodium carbonate, sodium dithionite, sodium chloride, sodium sulfate, magnesium sulfate, hydrochloric acid 32%, triethylamine, 1,4-dioxane, dimethyl formamide, dimethylsulfoxide, dichloromethane, methanol, petroleum spirit, acetone, dimethylacetamide, and diethyl ether were obtained from Chem-Supply and also used as received. Milli-Q water with a resistivity of >18 MΩ·cm was obtained from an in-line Millipore RiOs/Origin water purification system.

Chemical Characterizations

Nuclear magnetic resonance

All ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopies were performed by a Bruker 300 or 400 MHz spectrometer using deuterated solvents (obtained from Cambridge Isotope Laboratories) as reference solvents and at a sample concentration of ca. 10−20 mg mL^{-1}.

Gel permeation chromatography

Gel permeation chromatography (GPC) was carried out on a Shimadzu liquid chromatography system equipped with an SIL-20A auto-injector, a Polymer Laboratories 5.0 μm bead-size guard column (50 × 7.5 mm^2) followed by three linear PL (Styragel) columns (10^5, 10^4 and 10^3

Å) and an RID-10A differential refractive-index (RI) detector operating at 50 °C. Dimethylacetamide (containing 0.03% w/v LiBr and 0.05% w/v 2,6-dibutyl-4-methylphenol) was used as the eluent at a flow rate of 1 mL min^{-1}. The system was calibrated with poly(methyl methacrylate) standards with molecular weights from 200 to 10^6 g mol^{-1}.

Dynamic light scattering

Dynamic light scattering was performed using a Malvern Zetasizer Ultra–Red (Malvern Panalytical). Compounds were analyzed at 25 °C in deionized water, where the sample solution was filtered through a 0.45 μm pore size filter and pipetted into a disposal square polystyrene cuvette (DTS0012, Malvern Panalytical) for analysis. The critical micelle concentration (CMC) was determined by the mean count rate of the scattered light against serially diluted samples in which the measurement parameters of DLS (e.g., the attenuator and cell position) were fixed. The segmental linear regression was performed using Graphpad Prism.

Ultraviolet-visible (UV-vis) spectroscopy

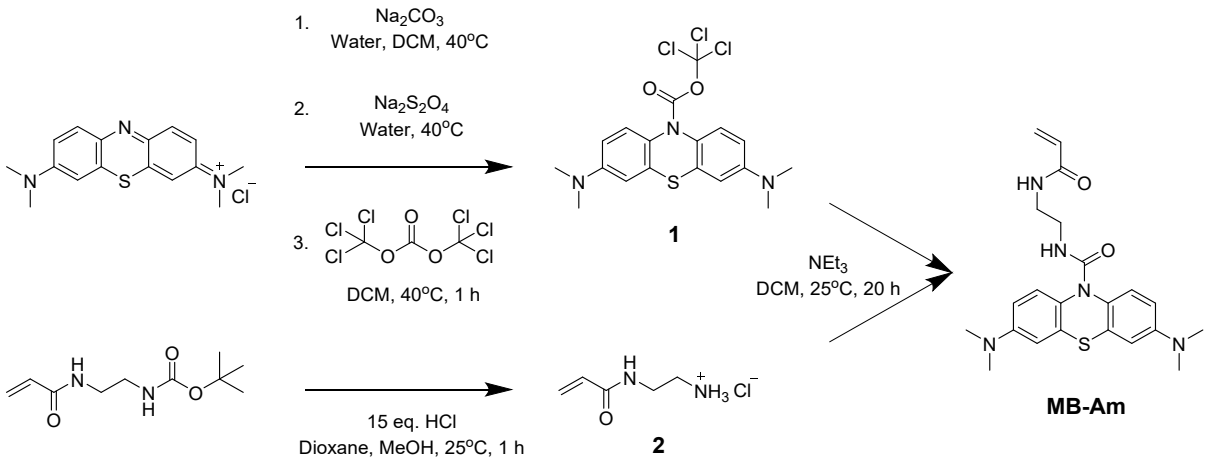
UV-vis absorption spectra were recorded using a CARY 300 Spectrophotometer (Agilent) from 300 to 800 nm at a scan rate of 600 nm min^{-1}. Spectra were acquired in 0.4 mL glass cuvettes with 1 mm pathlength.

Fluorescence spectroscopy

Fluorescence spectra were recorded using a RF-6000 Spectrofluorometer (Shimadzu) at a scan rate of 200 nm min^{-1}. The excitation and emission bandwidth were set to 1.5 nm and 3 nm, respectively. Spectra were acquired in 0.7 mL quartz cuvettes with 2 mm pathlength.

Chemical Synthesis

Methylene blue-functionalized ethyl acrylamide monomer MB-Am



Scheme S1. Synthesis pathway for the methylene blue-functionalized acrylamide monomer **MB-Am**.

The synthesis of the methylene blue-functionalized acrylamide monomer **MB-Am** proceeded according to Scheme S1 where both compounds **1** and **2** were synthesized separately before reacting together to yield the photosensitive monomer.

To make compound **1**, methylene blue hydrate (2.50 g) was first mixed with water (25 mL) in a flask and bubbled with nitrogen gas at 40 °C. Sodium carbonate (3.32 g, 31.3 mmol) was then added to the flask, followed by dichloromethane (12.5 mL) and more water (35 mL) – keeping note that the solution was continuously bubbled with nitrogen at 40 °C. Sodium dithionite (5.45 g, 31.3 mmol) was then added to the reaction mixture, in which the color of the solution immediately turned yellow along with the formation of some precipitates. Triphosgene (1.39 g, 4.68 mmol) solution in dichloromethane (20 mL) was added to the flask, followed by more dichloromethane (30 mL). The reaction mixture was stirred at 40 °C for 1 h under nitrogen bubbling. At the end of the reaction, the nitrogen bubbling was stopped, and the flask was removed from the oil bath. Ice water (100 mL) was added, and the entire content in

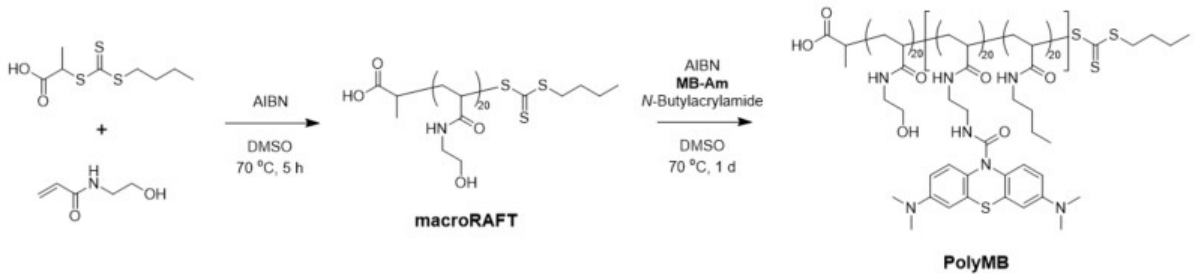
the flask was extracted with dichloromethane (60 mL). The organic fraction was collected and set apart. The aqueous phase was extracted once more with dichloromethane (60 mL) and the organic fractions were combined, dried over sodium sulfate, filtered and concentrated in vacuo. The crude product was further purified by column chromatography on silica gel using petroleum spirit:dichloromethane 2:3 volume ratio solvent system as the eluent (R_f ~ 0.4) to yield compound **1** as a white solid powder (2.70 g). The compound was used immediately in the next step as it is hygroscopic and slowly turns blue under ambient conditions.

In parallel, compound **2** was made by simply treating *N*-[2-(Boc-amino)ethyl]acrylamide (1.55g, 7.2 mmol), which was dissolved in 1,4-dioxane (8 mL) and methanol (2 mL), with HCl solution (27 mL of 4 M in 1,4-dioxane, 109 mmol). The contents were left to stir at 25 ^{o}C for 1 h during which white precipitates formed. At the end of the reaction, diethyl ether (40 mL) was added to further precipitate the product. The precipitates were recovered by centrifugation and redissolved in methanol (7 mL), and reprecipitated once more in diethyl ether (70 mL). The solids were recovered by centrifugation and dried in vacuo to yield compound **2** as a white solid (0.81 g, 75 mol% yield). The compound is hygroscopic and was used soon after in the next step.

Finally to make the methylene blue monomer, both compounds 1 (0.55 g, 3.7 mmol) and 2 (1.28 g, 3.7 mmol) were weighed into a flask, followed by the addition of dichloromethane (50 mL). To this suspension was added triethylamine (2.6 mL, 18.3 mmol). The solution became clear and left to stir at 25 ^{o}C for 20 h. Thereafter, the contents were washed successively against 0.1 M HCl (30 mL), water (30 mL), and brine (30 mL). The organic fraction was dried over magnesium sulfate, filtered, and concentrated in vacuo. The product was further purified by column chromatography on silica gel using a gradient solvent system as the eluent, starting from pure dichloromethane to dichloromethane with 1 vol% methanol, and to dichloromethane with 2.5 vol% methanol (R_f ~ 0.17). The purified monomer MB-Am was obtained as a light

blue solid (1.24 g, 79 mol% yield). ^{1}H NMR (400 MHz, DMSO-d_6, 25 ^{o}C): δ_H (ppm) = 8.12-8.14 (br s, 1H, CH2=CH-C(=O)-N*H*), 7.30-7.33 (d, 2H, N-C-C*H*), 6.63-6.72 (m, 4H, N-C-CH-C*H*-C-C*H*), 6.08-6.28 (m, 3H, H-C*H*=C*H*-C(=O)-NH-$(CH_2)_2$-N*H*), 5.59-5.63 (dd, 1H, *H*-CH=CH), 3.19-3.25 (dt, 4H, NH-$(CH_2)_2$-NH), 2.88 (s, 12H, N-$(CH_3)_2$). ^{13}C NMR (400 MHz, DMSO-d_6, 25 ^{o}C): δ_C (ppm) = 165.35, 155.77, 148.93, 133.59, 132.30, 128.85, 127.81, 125.47, 111.62, 110.75, 40.68, 40.28, 40.00, 39.72.

Red light-responsive antimicrobial methylene blue polymer PolyMB



Scheme S2. Synthesis of methylene blue polymer **PolyMB** via RAFT polymerization.

The red light-responsive methylene blue polymer **PolyMB**, which is a diblock copolymer, was synthesized via two consecutive RAFT polymerizations (Scheme S2). The first block is a homopolymer made of *N*-hydroxyethyl acrylamide whereas the second block is a random copolymer of **MB-Am** and *N*-butylacrylamide.

To make the first block **macroRAFT** agent, *N*-hydroxyethyl acrylamide (1.00 g, 8.7 mmol), 2-(butylthiocarbonothioylthio)propanoic acid (83 mg, 0.35 mmol), AIBN (23 mg, 0.14 mmol) and dimethylsulfoxide (8.7 mL) were firstly mixed in a flask and deoxygenated by bubbling with nitrogen for 30 min. The reaction mixture was then stirred at 70 ^{o}C for 5 h under nitrogen atmosphere before cooling in an ice bath. The mixture was diluted with methanol (7 mL) and precipitated into diethyl ether (100 mL). The precipitate was isolated by centrifugation, redissolved in methanol (ca. 15 mL), and precipitated once more in diethyl ether (100 mL),

followed by drying in vacuo to yield the **macroRAFT** polymer as a light yellow solid (ca. 690 mg yield). It is noteworthy that the monomer conversion was ca. 80% by ^{1}H NMR analysis. GPC (dimethylacetamide, 50 °C): M_n = 6400 g mol^{-1}, $Đ$ = 1.17.

The chain extension was performed by initially dissolving the **macroRAFT** polymer (58 mg, 0.025 mmol) in dimethylsulfoxide (1 mL), followed by the addition of **MB-Am** monomer (213 mg, 0.50 mmol), *N*-butylacrylamide (64 mg, 0.50 mmol) and AIBN (2 mg, 0.010 mmol). The reaction mixture was deoxygenated by bubbling with nitrogen for 15 min, and then stirred at 70 °C for 24 h under nitrogen atmosphere before cooling in an ice bath. The mixture was precipitated directly into diethyl ether (10 mL) and the precipitate was isolated by centrifugation. The solids were redissolved in ca. 1 mL of acetone:methanol 1:1 volume ratio solvent system and precipitated into diethyl ether (10 mL). The precipitation step was repeated once more before drying the polymer in vacuo. The obtained **PolyMB** was a blue solid product (ca. 300 mg yield). The monomer conversion was quantitative according to ^{1}H NMR analysis. GPC (dimethylacetamide, 50 °C): M_n = 14100 g mol^{-1}, $Đ$ = 1.21.

Red Light Photoreactor Setup

The red light LED array for 96-well plate (LEDA-R, Bio Research Center Co., Ltd.) was powered via a LED array driver (LAD-1, Bio Research Center Co., Ltd). The voltage of the array driver was fixed at 13 V to supply a sustainable and stable red light source (λ_{max} = 630 nm, 18 mW/cm^2).

Mechanism Investigation

Red light driven uncaging kinetic

The red light-driven uncaging kinetics of **PolyMB** (1.18 mM in H_2O) were monitored under photoirradiation (λ_{max} = 630 nm, 18 mW/cm^2) for 3 h using UV-vis spectroscopy. Initially, the

visible absorption spectrum of methylene blue was calibrated in water across a series of concentration gradients. Due to the self-aggregation of methylene blue in aqueous solution and the overlapping characteristic peaks of its dimer and mesomere forms, the calibration and linear fitting were conducted over a broad absorbance range (420 – 800 nm).[1] Subsequently, the uncaging kinetics of **PolyMB** were monitored in a sealed glass cuvette (0.4 mL, 1 mm path length). The cleavage rate was determined based on the established calibration curve for methylene blue. Given the hydrophobic nature of **MB-Am**, the calibration and uncaging kinetics of the monomer (**MB-Am**) were similarly studied but using acetonitrile as the solvent instead of water.

Determination of singlet oxygen ($^1O_2$) formation

The formation of $^1O_2$ was monitored under photoirradiation (λ_{max} = 630 nm, 18 mW/cm^2) over 20 min via the trapping of $^1O_2$ with 9,10-dimethylanthracene (9,10-DMA) in a model experiment.[2] Briefly, the solution (3.6 μM **PolyMB**, 1 mM 9,10-DMA, and 350 μL DMF) was prepared in a glass cuvette (0.4 mL, 1 mm pathlength) and sealed. Under red light irradiation, the characteristic peak of 9,10-DMA (absorption at ~380 nm) was monitored by UV-vis spectroscopy at several time points. The decreasing absorption of 9,10-DMA reflected the generation and quenching of $^1O_2$.

Determination of superoxide ($O_2^{\bullet-}$) formation

The formation of $O_2^{\bullet-}$ was monitored under photoirradiation (λ_{max} = 630 nm, 18 mW/cm^2) over 30 min via the trapping of $O_2^{\bullet-}$ with nitrotetrazolium blue chloride (NBT) in a model experiment.[3] Briefly, the solution (3.6 μM **PolyMB**, 50 μM NBT, and 350 μL deionized water) was prepared in a glass cuvette (0.4 mL, 1 mm pathlength) and sealed. Under red light

irradiation, the characteristic peak of NBT (absorption at ~260 nm) was recorded by UV-vis spectroscopy. The lack of change in absorption of NBT reflected the absence of $O_2^{\bullet-}$.

Photo-induced protein damage study

The protein damage experiment was carried out under photoirradiation (λ_{max} = 630 nm, 18 mW/cm²) over 60 minutes. Briefly, a solution containing **PolyMB** (8 μg mL^{-1}), bovine serum albumin (BSA, 2 mg mL^{-1}), and PBS (600 μL) was prepared in a quartz fluorometer cuvette (0.7 mL, 2 mm path length) and sealed. During red light irradiation, the intrinsic protein fluorescence (λ_{ex} = 280 nm, λ_{em} = 341 nm) was monitored using a fluorescence spectrophotometer. The decreasing BSA fluorescence indicated the occurrence of red light-induced protein damage caused by **PolyMB**.

Biological Experiments

Minimum inhibitory concentration (MIC) determination

The MICs were determined by the broth microdilution method against two gram-negative pathogens (*Pseudomonas aeruginosa* ATCC 27853 and Escherichia coli ATCC 25922) and one gram-positive pathogen (*Staphylococcus aureus* ATCC 29213) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines to evaluate the bacteriostatic efficacy of the compounds. Briefly, a single bacterial colony was cultured in 10 mL of Mueller-Hinton Broth (MHB) at 37 °C with 180 rpm shaking overnight. Subsequently, a subculture was prepared from the overnight culture by diluting 1:100 in 10 mL MHB and allowed to grow to mid-log phase. The grown subculture was then diluted to the appropriate concentration for the MIC test. A twofold dilution series of 100 μL of compounds solution in PBS were added into 96-well microplates (Costar, Corning), followed by the addition of 100 μL of the subculture suspension. The final concentration of bacteria in each well was ca. 5 × 10^5 cells mL^{-1}. The

microplate was then subjected to red light irradiation (λ_{max} = 630 nm, 18 mW/cm^2) for 2 h. A parallel control plate was also prepared as above but without irradiation. After incubating the plates at 37 °C for 20 h, the absorbance at 600 nm was measured using a microtiter plate reader (FLUOstar Omega, BMG Labtech). MIC values were defined as the lowest concentration of sample that showed no visible bacteria growth and inhibited more than 90% bacteria growth. Positive controls without compounds and negative controls without bacteria were included. All assays included duplicates and were repeated in at least three independent experiments.

Planktonic bacteria killing study

The bactericidal efficacy of the compounds was evaluated against the Gram-negative bacteria *E. coli* in PBS. The bacterial suspension was prepared in the same manner as with the MIC assay, albeit using PBS as the dilution media in lieu of MHB. Two initial concentrations (ca. 5 × 10^6 and 1 × 10^9 cells mL^{-1}) of bacteria in the vial were challenged in this assay. A solution containing either **PolyMB** (8 μg mL^{-1}) or methylene blue (4 μg mL^{-1}) was mixed with bacteria suspension in a glass vial and photoirradiated (λ_{max} = 630 nm, 18 mW/cm^2) for 1 h. The viability of planktonic cells was monitored at several time points via a drop plate method where the planktonic cells were serially diluted in sterile PBS and plated onto Luria Bertani (LB) agar. After subjecting the plates to 24 h of incubation at 37 °C, bacteria colonies were counted and colony forming unit (CFU) analysis was performed. All assays included duplicates and were repeated in at least two independent experiments.

Inner membrane permeability study

An inner membrane disruption kinetic assay with temporal resolution was applied based on the propidium iodide (PI) staining method.[4] To initiate the assay, a concentrated bacteria suspension of *E. coli* in PBS (ca. 4 × 10^8 cells mL^{-1}) was prepared via centrifugation (2000 *g*,

10 min) and subsequent resuspension. The compounds at selected concentration (2 × MIC) were then loaded on a flat-bottom, black cell-culture-grade 96-well plate (Costar, Corning), followed by the addition of PI (5 μmol, Sigma-Aldrich) and bacteria suspension. Under photoirradiation (λ_{max} = 630 nm, 18 mW/cm^2), the fluorescence intensity was monitored for 30 min using a microtiter plate reader (FLUOstar Omega, BMG Labtech) with excitation and emission wavelengths of 544 nm and 620 nm, respectively. Negative (without antimicrobial) and positive (polymyxin B, 8 μg mL^{-1}) controls were included. The fluorescence intensity was normalized by the ratio to negative control. All assays included duplicates and were repeated in at least two independent experiments.

Light penetration antimicrobial study

The red light penetrating ability was examined using a chicken skin barrier model. Briefly, a piece of chicken skin (6.5 × 6.5 cm^2, cut from chicken thigh) was used to cover a glass vial, to which it was added bacterial suspension (ca. 5 × 10^6 cells mL^{-1}) and either **PolyMB** (8 μg mL^{-1}) or methylene blue (4 μg mL^{-1}). The chicken skin-covered glass vial was photoirradiated (λ_{max} = 630 nm, 18 mW/cm^2) for 1 h, and CFU analysis was conducted in the same manner as with the bactericidal assay. All assays included duplicates and were repeated in at least two independent experiments.

Mammalian cell viability assay

The mammalian cell viability study was determined by PrestoBlue (Thermo Fisher Scientific) assay on murine embryonic fibroblasts (MEF) CF-1 (ATCC SCRC-1040), which was kindly provided by the Cell Culture Facility of the Mark Wainwright Analytical Centre at UNSW. Using a cell culture incubator (Eppendorf CellXpert C170i), MEF cells were cultured to subconfluency at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Gibco)

that was supplemented by L-glutamine (2 mmol, Sigma-Aldrich) and fetal bovine serum (10% (v/v), Sigma-Aldrich). For the cell viability assay, MEF cells were subcultured twice and diluted to a final concentration of 5×10^4 cells mL^{-1} in DMEM. After adding 100 µL of cell suspension to each well of a flat-bottom, black cell-culture-grade 96-well plate (Costar, Corning), the plate was cultured for 20 h at 37 °C and 5% CO_2 to attach the cells. Then, the supernatant was aspirated, and 50 µL of fresh DMEM was added, followed by another 50 µL of the compounds in DMEM. Subsequently, the plate was incubated for 24 h at 37 °C and 5% CO_2. The fluorescence was measured (excitation 560 nm and emission 590 nm) using a microplate reader (CLARIOstar, BMG Labtech) after adding 10 µL of PrestoBlue agent in each well and culturing for 1 h at 37 °C and 5% CO_2. Before adding PrestoBlue agent, the fluorescence background of the compounds was checked, which was negligible. The cell viability and IC_{50} curves were calculated by GraphPad Prism. The cellular morphology after treatment was recorded via a microscope (CMS GmbH, Leica). All assays included duplicates and were repeated in at least three independent experiments.

SUPPORTING RESULTS

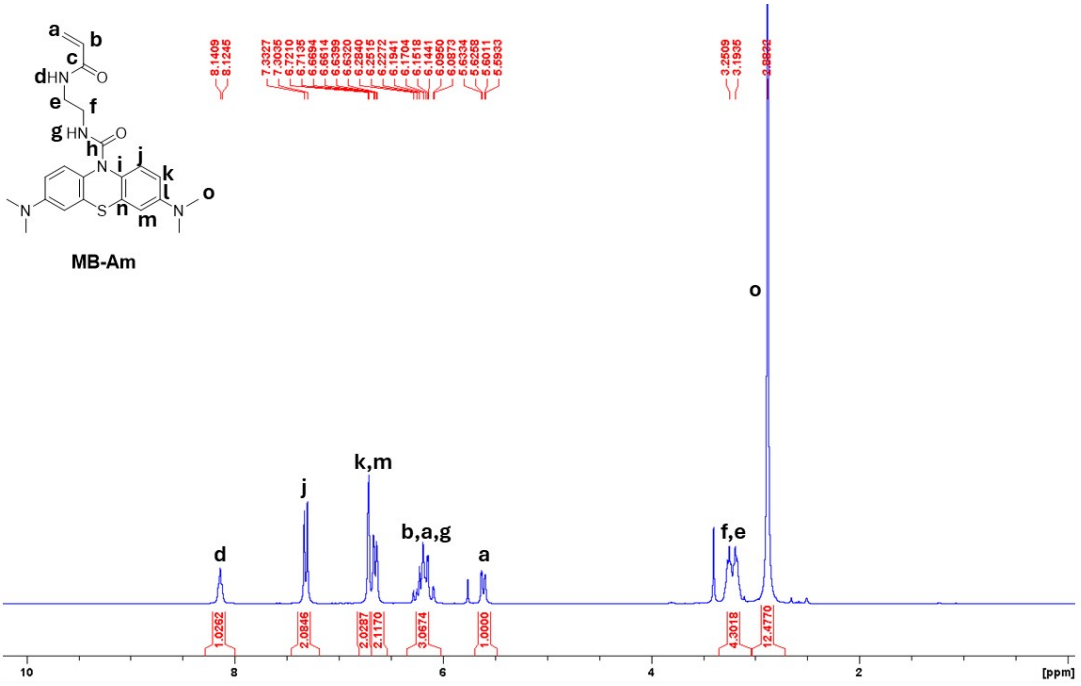


Figure S1. ^{1}H NMR spectra of monomer **MB-Am** in DMSO-d_6.

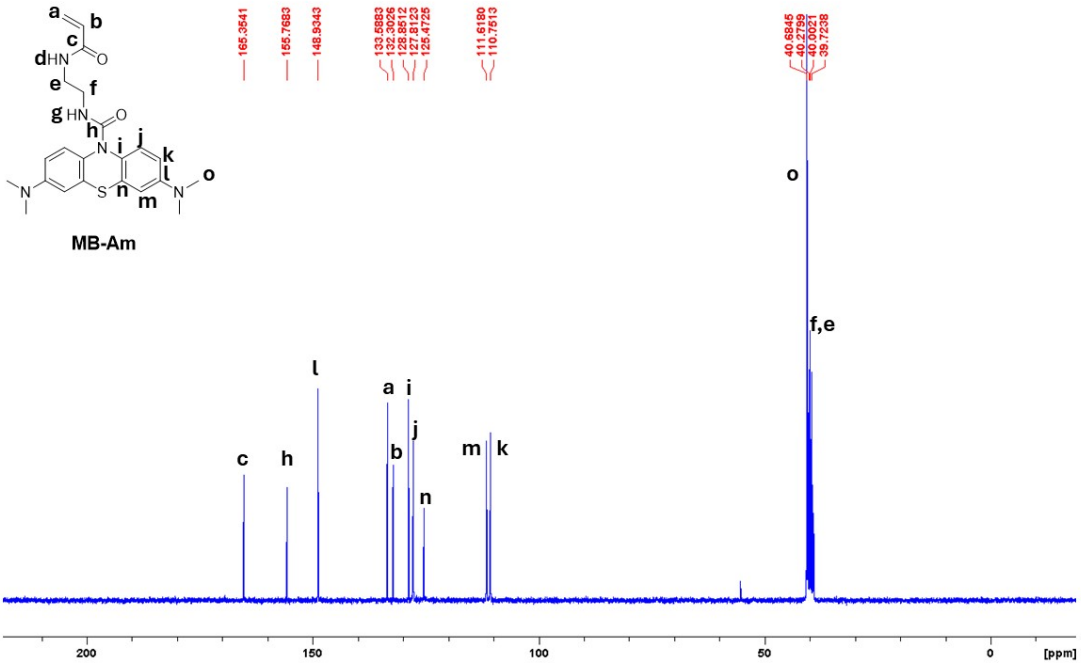


Figure S2. ^{13}C NMR spectra of monomer **MB-Am** in DMSO-d_6.

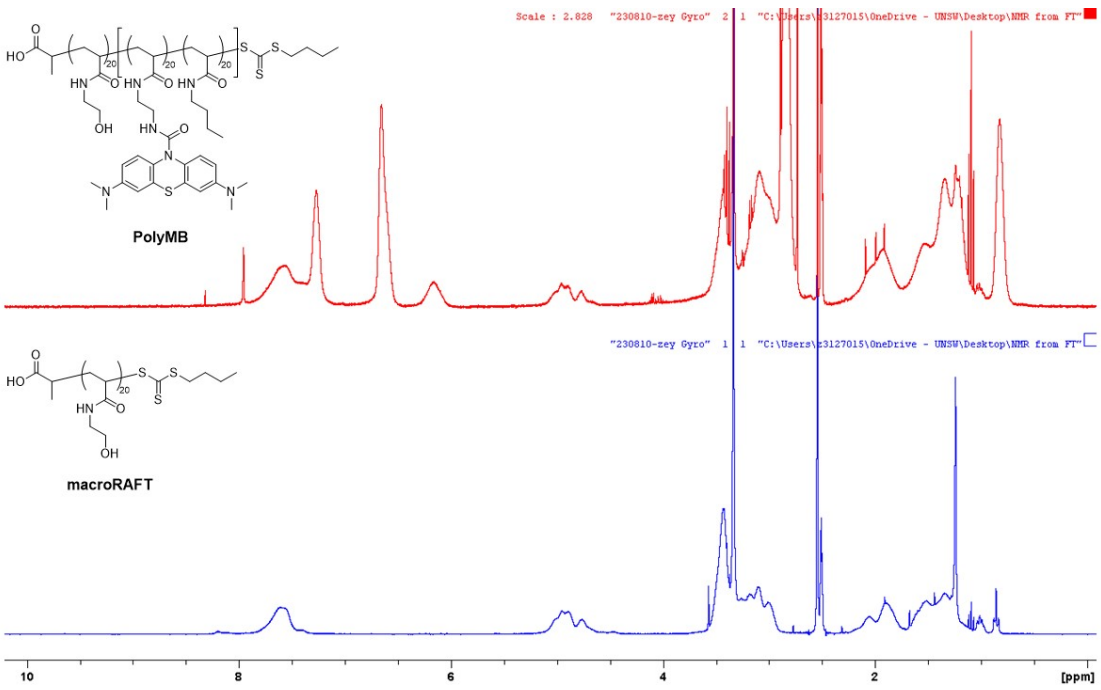


Figure S3. 1H NMR spectra of polymers in DMSO-d_6.

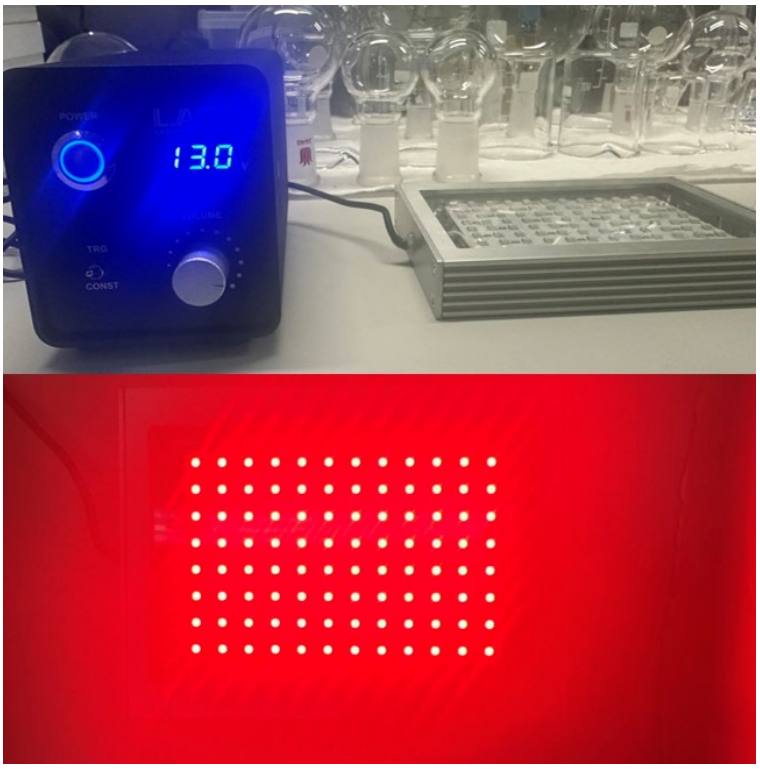


Figure S4. Red light photoreactor setup.

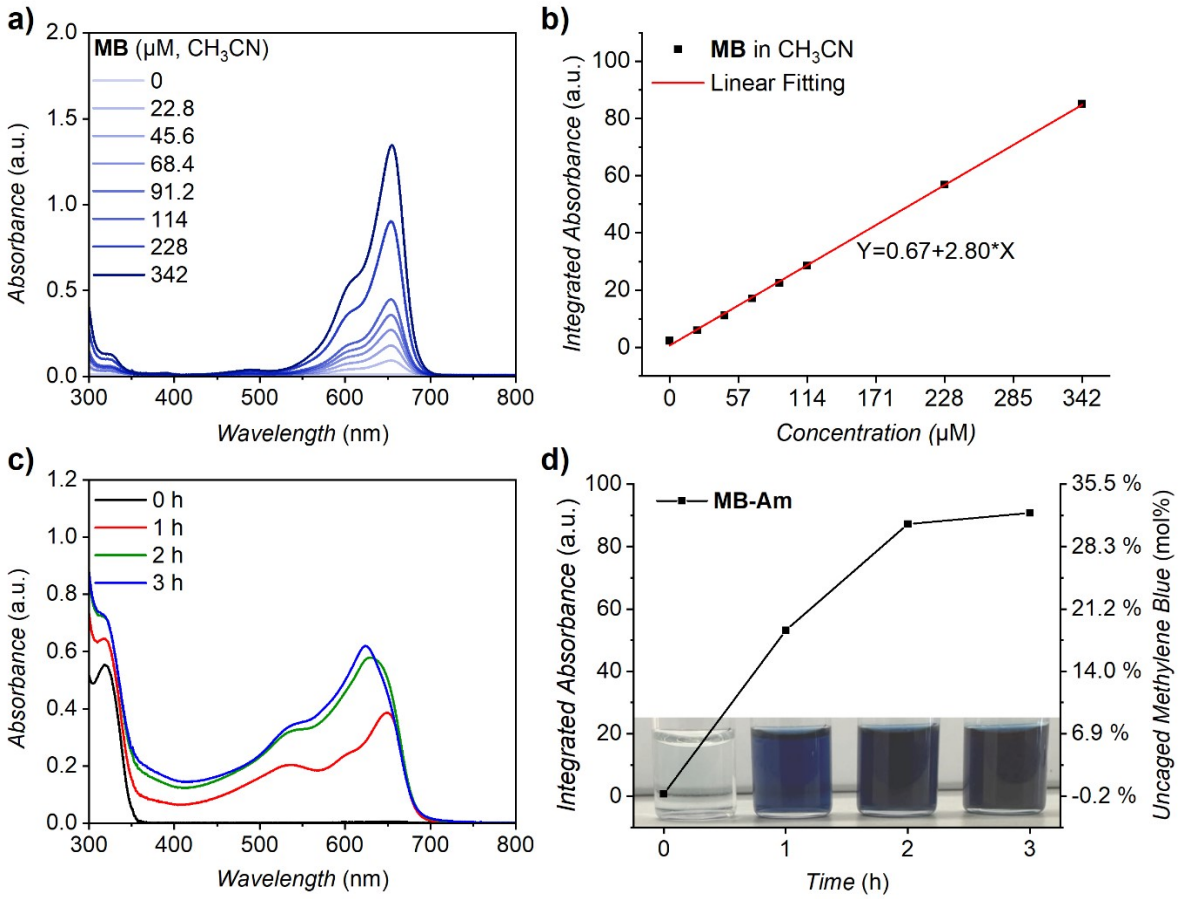


Figure S5. Red light photoinduced uncaging kinetics of **MB-Am** (1.14 mM in acetonitrile). a) UV-vis spectrum of different concentrations of methylene blue in acetonitrile; b) linear fitting of integrated UV absorbance of methylene blue (i.e. $\int_{420\,nm}^{800\,nm} Absorbance$) as a function of different concentrations of methylene blue solution in acetonitrile; c) UV-vis spectrum of **MB-Am** (1.14 mM) after different irradiation time (λ_{max} = 630 nm, 18 mW/cm^2); d) integrated UV absorbance (i.e. $\int_{420\,nm}^{800\,nm} Absorbance$) of photoirradiated **MB-Am** at different time points and the amount of uncaged methylene blue (mol%) calculated based on the linear fitting in b).

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