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Supporting Information for

Photo-Degradable Micelles for Co-delivery of Nitric Oxide and Doxorubicin

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Experimental section

Materials. *p*-Phenylenediamine, *o*-phenylenediamine, 2-bromoethanol, 3-hydroxybenzaldehyde, hexamethylene diisocyanate (HDI), doxorubicin hydrochloride (DOX·HCl), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), rhodamine B (RhB), sodium nitrite ($NaNO_2$), and sodium borohydride ($NaBH_4$) were purchased from Sinopharm Chemical Reagent Co., Ltd. Griess Reagent and PEG_{45}-OH were purchased from Sigma-Aldrich. PEG_{45}-OH was azeotropically distilled with toluene to remove the trace amount of water just prior to use. All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. and used as received without further purification unless otherwise stated. Water was deionized through a Milli-Q apparatus (Millipore) with a resistivity of 18.2 $M\Omega \cdot cm^{-1}$. Cell culture medium Dulbecco's modified eagle medium (DMEM) supplemented with 10 v/v% FBS and 1 v/v% antibiotic/antimycotic solution. Four to six week-old Balb/c mice were purchased from the Experimental Animal Center of Anhui Medical University. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of the University of Science and Technology of China (USTC) and were conducted in accordance with the Animal Care and Use Committee of USTC. Rhodamine B (RhB)-based NO probe was synthesized according to the literature procedure.[1]

Synthesis of Compound 1 (Scheme 2). Compound **1** was synthesized according to previously reported procedure with slight modifications.[2] Briefly, to a mixture of 3-hydroxybenzaldehyde (9.9 g, 81 mmol), anhydrous K_2CO_3 (14.6 g, 105.6 mmol), and KI (405 mg, 2.4 mmol) in anhydrous CH_3CN (120 mL), 2-bromoethanol (15 mL, 218 mmol) was added via a dropping funnel under nitrogen atmosphere at room temperature. The resultant mixture was refluxed at 90 ^oC for 36 h, cooled down to room temperature and filtered. The solid residues were washed with ethyl acetate (100 mL × 3). The organic phase was combined and was concentrated on a rotary evaporator. After that, the crude product was dispersed with water (80 mL) and extracted with ethyl acetate (100 mL × 2). The combined organic layer was dried over anhydrous Na_2SO_4, and the crude product was further

purified by column chromatography (EtOAc/petroleum ether = 1/2, v/v), yielding compound **1** as a white solid (4.6 g, yield: 34%). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, Figure S1): 9.98 (s, 1H), 7.58 – 7.50 (m, 2H), 7.47 – 7.40 (m, 1H), 7.34 – 7.24 (m, 1H), 4.93 (t, *J* = 5.5 Hz, 1H), 4.07 (dd, *J* = 5.4, 4.5 Hz, 2H), 3.74 (*J* = 5.4, 4.4 Hz, 2H). HR ESI-MS (Figure S2): m/z: Calcd. for $C_9H_{10}NaO_3$ $[M + Na]^+$: 189.0522; found: 189.0521.

Synthesis of Compound 2 (Scheme 2). Compound **1** (517 mg, 3.1 mmol) was dissolved in anhydrous ethanol (10 mL), and *p*-phenylenediamine (161 mg, 1.5 mmol) solution (dissolved in 8 mL anhydrous ethanol) was added dropwise. The mixture was stirred overnight at room temperature. The mixture was filtered, and the solid residues were washed with anhydrous ethanol, yielding compound ***2*** as a pale yellow solid (519 mg, yield: 86%). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm; Figure S3): 8.66 (s, 2H), 7.56 – 7.49 (m, 4H), 7.44 (t, *J* = 8.0 Hz, 2H), 7.37 (s, 4H), 7.12 (dd, *J* = 8.1, 2.4 Hz, 2H), 4.90 (t, *J* = 5.5 Hz, 2H), 4.07 (t, *J* = 4.9 Hz, 4H), 3.76 (dd, *J* = 10.2, 5.2 Hz, 4H). HR ESI-MS (Figure S4): m/z Calcd. for $C_{24}H_{25}N_2O_4$ $[M + H]^+$: 405.1809; Found: 405.1811.

Synthesis of Compound 3 (Scheme 2). Compound **2** (404 mg，1 mmol) was dispersed in a mixture of ethanol and THF (20 mL, 1/1, v/v). Sodium borohydride (300 mg, 8 mmol) was then added and stirred at room temperature. The reaction was monitored by thin-layer chromatography (TLC) until completion. The residues were successively washed with water (10 mL × 3), anhydrous ethanol (10 mL × 3), and diethyl ether (10 mL × 3), yielding compound ***3*** as a pale yellow solid (353 mg, yield: 87 %). ^{1}H NMR (400 MHz, DMSO-d$_6$, δ, ppm; Figure S5): 7.18 (t, *J* = 7.8 Hz, 2H), 6.89 (d, *J* = 7.8 Hz, 4H), 6.79 – 6.71 (m, 2H), 6.38 (s, 4H), 5.37 (t, *J* = 6.1 Hz, 2H), 4.83 (t, *J* = 5.5 Hz, 2H), 4.10 (d, *J* = 6.1 Hz, 4H), 3.93 (t, *J* = 5.0 Hz, 4H), 3.68 (dd, *J* = 10.3, 5.2 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6, δ, ppm; Figure S6): 159.19, 143.19, 140.52, 129.64, 119.88, 114.26, 113.83, 112.63, 69.74, 60.06, 48.05. HR ESI-MS (Figure S7): m/z: Calcd. for $C_{24}H_{28}N_2NaO_4$ $[M + Na]^+$: 431.1941; found: 431.1930.

Synthesis of Compound 4 (Scheme 2). Sodium nitrite (1 g, 14.5 mmol) was

dispersed in a mixture of water and THF (20 mL, 1/1, v/v) and compound ***3*** (300 mg, 0.74 mmol) solution (dissolved in 30 mL glacial acetic acid) was added dropwise. The mixture was stirred overnight at room temperature followed by concentration on a rotary evaporator. After filtration, the crude product was successively washed with water (10 mL × 3), anhydrous ethanol (10 mL × 3), and diethyl ether (10 mL × 3) to afford compound ***4*** as a pale yellow solid (306 mg, yield: 89 %). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm; Figure S8a): 7.79 (s, 4H), 7.19 (t, J = 7.9 Hz, 2H), 6.81 (dd, J = 8.2, 2.2 Hz, 2H), 6.66 – 6.57 (m, 4H), 5.33 (s, 4H), 4.83 (s, 2H), 3.90 (t, J = 4.9 Hz, 4H), 3.66 (d, J = 4.7 Hz, 4H).^{13}C NMR (101 MHz, DMSO-d_6, δ, ppm; Figure S8b): 159.36, 140.35, 136.38, 130.35, 120.96, 119.39, 113.87, 113.59, 70.09, 60.18, 46.33. HR ESI-MS (Figure S8c): m/z: Calcd. for $C_{24}H_{26}N_4NaO_6$ $[M + Na]^+$: 489.1745; found: 489.1726. HPLC (Figure S8d)： t_R = 10.63 min.

Synthesis of PEG-b-PNORM-b-PEG Triblock Copolymer Through Polycondensation Reaction (Scheme 2). Compound ***4*** (137 mg, 0.25 mmol) and freshly distilled HDI (44 mg, 0.26 mmol) were dissolved in 2 mL of anhydrous dimethyl sulfone (DMSO). Then, two drops of dibutyltin dilaurate (DBTL) were added. The mixture was stirred at room temperature for 2 h and dried PEG_{45}-OH (251 mg, 0.12 mmol,) was added. After addition, the mixture was stirred at room temperature for an additional 30 h. After that, the mixture was precipitated into a mixed solution of diethyl ether and dichloromethane (40 mL, v/v = 4/1) three times. After drying in a vacuum oven overnight at room temperature, the triblock copolymer, PEG-*b*-PNORM-*b*-PEG, was obtained as a yellowish solid (200 mg; yield: 54 %, Figure S9).

Photolysis of compound 4. The photolysis process of compound ***4*** was monitored by UV-vis spectroscopy. Briefly, compound ***4*** (20 μM) was dissolved in a mixture of DMSO and water (1/1, v/v). The evolution of the absorbance changes was continuously monitored under 410 nm light irradiation (9.5 mW/cm^2) for varying times (0-20 min). Meanwhile, the NO release from compound **4** under 410 nm light irradiation was quantified by Griess reagent using a standard calibration curve (Figures S12 and S14).

Self-Assembly of PEG-b-PNORM-b-PEG Triblock Copolymers. PEG_{45}-*b*-$PNORM_5$-*b*-PEG_{45} triblock copolymer was dissolved in 1,4-dioxane solution (1 mg/mL) under vigorous stirring (1000 rpm/min). Deionized water (8 mL) was then injected in one shot, and the mixture was stirred for 30 min at room temperature. Afterward, 1,4-dioxane was removed by dialysis (MWCO 3.0 kDa) against deionized water.

Photolysis of PEG-b-PNORM-b-PEG Micelles. The photolysis process of the micelles was monitored by UV-vis spectroscopy, dynamic light scattering (DLS), and transmission electron microscopy (TEM). Briefly, the colloidal dispersion (PEG_{45}-*b*-$PNORM_5$-*b*-PEG_{45}, 0.1 g/L) was added to a standard quartz cuvette (optical path length: 1 cm) and irradiated with a 410 nm LED lamp (9.5 mW/cm^2). At predetermined intervals, the absorption spectra of the irradiated dispersion were recorded. Meanwhile, the sizes of the micelles after irradiation were measured by DLS, and the morphological transitions were observed by TEM. The photo-triggered NO release was monitored by the Griess assay.

DOX-Loading and Photo-Triggered DOX Release. Doxorubicin hydrochloride (DOX·HCl, 10.0 mg) was deprotonated by mixing with triethylamine (TEA, 2 mg) in 1 mL of dry DMSO, followed by stirring for 4 h.[3] Pure water (8 mL) was quickly added to the 1,4-dioxane solution (1 mL) containing 4 mg/mL of PEG_{45}-*b*-$PNORM_5$-*b*-PEG_{45} and 1 mg/mL of DOX. Then, the mixture was stirred for 0.5 h at room temperature. Thereafter, 1,4-dioxane was removed by dialysis (MWCO 3.0 kDa) against deionized water. To determine drug loading efficiency (DLE) and drug loading content (DLC), DOX-loaded suspension of PEG_{45}-*b*-$PNORM_5$-*b*-PEG_{45} micelles (0.4 g/L, 1mL) was lyophilized and then re-dispersed in DMSO (10 mL). Fluorescence intensity of the DMSO solution was measured, and the DOX content was calculated against a standard calibration curve established in DMSO (Figure S20b). The DLC and DLE were determined to be 3.3% and 13.4%, respectively (Figure S20).

For photo-triggered mediated DOX release, 800 μL of the aqueous dispersion of DOX-loaded micelles (0.4 g/L) was irradiated with 410 nm light (9.5 mW/cm^2) for

varying durations. The untreated and irradiated micelles were then subjected to dialyzed against deionized water (10 mL). At predetermined intervals, freshwater was replenished, and the dialysate was lyophilized and dissolved in DMSO for fluorescence analysis. The cumulative release amounts of DOX were quantified by fluorescence intensity at 562 nm against the calibration curve (Figure S20).

Cytotoxicity Assessment of DOX-free and DOX-loaded PEG-b-PNORM-b-PEG Micelles. Alamar blue assay was used to evaluate cell cytotoxicity in vitro. HeLa cells and DOX-resistant MCF-7 cells (e.g., MCF-7/DOX) were chosen to evaluate cell cytotoxicity of empty or DOX-loaded PEG-*b*-PNORM-*b*-PEG micelles. HeLa cells were cultured in DMEM complete medium, while MCF-7/DOX cells were cultured in DMEM complete medium that contained 500 ng/mL DOX to maintain the DOX resistance. For cell cytotoxicity experiments, cells were seeded in 96-well plates in 100 μL of DMEM complete medium at a density of 10,000 cells/well. After overnight incubation, the cells were treated with empty or DOX-loaded micelles at varying concentrations (0.01, 0.02, 0.05, 0.1, 0.2, 0.4 g/L). The micelle-treated cells were then irradiated with an LED lamp (410 nm, 9.5 mW/cm^2) for 0, 10, or 20 min. After irradiation, the cells were further cultured for another 24 or 48 h, the medium in each well was removed and replaced by 100 μL of fresh DMEM. Then, Alamar blue reagent (10 μL, 5 mg/mL, Thermo Fisher) was added to each well, and the cells were incubated for 4 h at 37 ^{o}C. Finally, the fluorescence emission at 590 nm (excitation at 550 nm) was recorded on a plate reader (Thermo Fisher Scientific).

The cell viability was calculated using the following equation:

$$\text{Cell Viability}/\% = (FL_{590,\ micelles} - FL_{590,\ blank})/(FL_{590,\ control} - FL_{590,\ blank}) \times 100\%$$

Where $FL_{590,\ micelles}$ is the fluorescence intensity of the wells incubated with micelles; $FL_{590,\ control}$ is the fluorescence intensity of the solution of the wells with only media without micelles and $FL_{590,\ blank}$ is the fluorescence intensity of wells with culture media without cells.

Photo-Mediated Intracellular NO Release*.* The intracellular NO release was monitored by the fluorescence changes of the NO-sensitive rhodamine B

(RhB)-based probe.[1] Firstly, HeLa cells were seeded in a single-well plate at an initial density of 100,000 cells/well in 1 mL of DMEM medium. After overnight incubation, cells were treated with micelles (0.4 g/L) for 6 h followed by the addition of RhB probe (100 μM) or not. After that, the cells were further incubated for 1 h and irradiated with an LED lamp (410 nm, 9.5 mW/cm^2) for 20 min. Thereafter, cells were stained with Hoechst 33342 (5 μg/mL) and incubated for 20 min, followed by washing with PBS buffer three times and were observed with a Leica SP-5Confocal Laser Scanning Microscopy (CLSM). The blue channel was excited at 405 nm and collected at 410-500 nm. The red channel was excited at 514 nm and collected at 520-700 nm.

***In vivo Subcutaneous Observation of NO Release*.** All animals received care in strict accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals by the Laboratory Animal Center in the University of Science and Technology of China (USTC) and all the experimental procedures in this study were approved by the USTC Animal Care and Use Committee. At first, dorsal hair of 4-6 week-old Balb/c mice was shaved. Aqueous dispersion (100 μL, 0.4 g/L) of PEG_{45}-*b*-$PNORM_5$-*b*-PEG_{45} micelles mixed with rhodamine probe (100 μM) was injected into left hind limbs of mice. PBS buffer (100 μL, 10 mM) mixed with rhodamine probe (100 μM) was injected into right hind limbs of mice as control.- After that, the mice were treated with or without 410 nm irradiation (31.5 mW/cm^2) for varying times, followed by recording the changes of fluorescence on an IVIS® Lumina III imaging system (Ex: 560 nm, Em: 620 nm). All the animal experiments were approved by the Committee on the Ethics of Animal Experiments of the University of Science and Technology of China (USTC) and were conducted in accordance with the Animal Care and Use Committee of USTC.

Characterizations.

Nuclear magnetic resonance (NMR) spectra were performed on a Bruker Avance 400 MHz spectrometer operated in the Fourier transform mode. Deuterated dimethyl sulfone (DMSO-d_6) was used as the solvent. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) experiment was performed on Thermo

Scientific LTQ Orbitrap Mass Spectrometer equipped with an electrospray interface. High-performance liquid chromatography (HPLC) analysis was performed with a Shimadzu HPLC system, equipped with an LC-20AP binary pump, an SPD-20A UV-vis detector, and a Symmetry C18 column. Fluorescence spectra were recorded on an F-4600 (Hitachi) spectrofluorometer. Dynamic laser light scattering (LLS) measurements were performed on a commercial spectrometer (ALV/CGS-3, Langen/Hessen, Germany). A laser (λ_0 = 632.8 nm) was used as the light source. The scattered light was collected at a fixed angle of 90^o, and the duration of each measurement was 5 min. All data were averaged over three consecutive measurements. UV/Vis spectra were recorded on a TU-1910 double-beam UV-vis spectrophotometer (Puxi General Instrumental Company, China). Confocal laser scanning microscopy (CLSM) images were observed using a Leica TCS SP5 microscope. Transmission electron microscopy (TEM) measurements were conducted on a JEM-2100 electron microscope (JEOL Ltd.), and the acceleration voltage was 200 kV. Samples for TEM tests were prepared by placing 10 μL of micelles dispersion on copper grids and stained with 2 wt% phosphotungstic acid. Gel permeation chromatography (GPC) equipped with Waters 1515 pump and Waters 2414 differential refractive index detector (set at 30 ^{o}C) was used to determine the molecular weights and molecular weight distributions of polymers. A series of polystyrene standards with low polydispersities were used for calibration. The eluent was THF, and the flow rate was set to 1.0 mL/min. A JEOL JES-FA200 spectrometer (300 K, 9.063 GHz, X-band) was used to record the electron paramagnetic resonance (EPR) spectra at room temperature. The following parameters were used for the measurements, microwave power: 1 mW; sweep width: 319.3 to 329.3 mT, modulation frequency: 100 kHz, and modulation amplitude: 0.35 mT.

References

1. H. Zheng, G. O. Shang, S. Y. Yang, X. Gao and J. G. Xu, *Org Lett*, 2008, **10**, 2357-2360.
2. D. Kalia, S. P. Pawar and J. S. Thopate, *Angew. Chem. Int. Ed.*, 2017, **56**,

1885-1889.
3. Z. Wang, T. Luo, R. L. Sheng, H. Li, J. J. Sun and A. Cao, *Biomacromolecules*, 2016, **17**, 98-110.

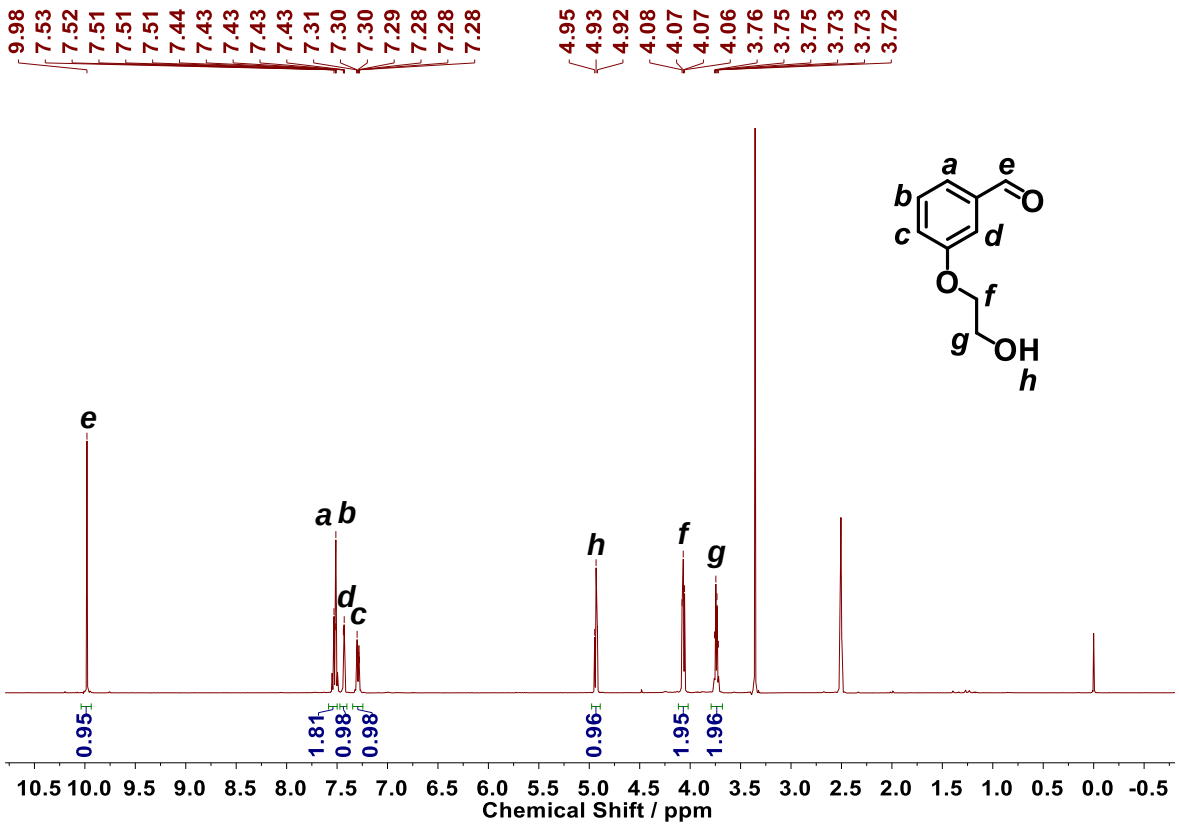

Figure S1. ^{1}H NMR spectrum recorded in DMSO-d_6 for compound ***1***.

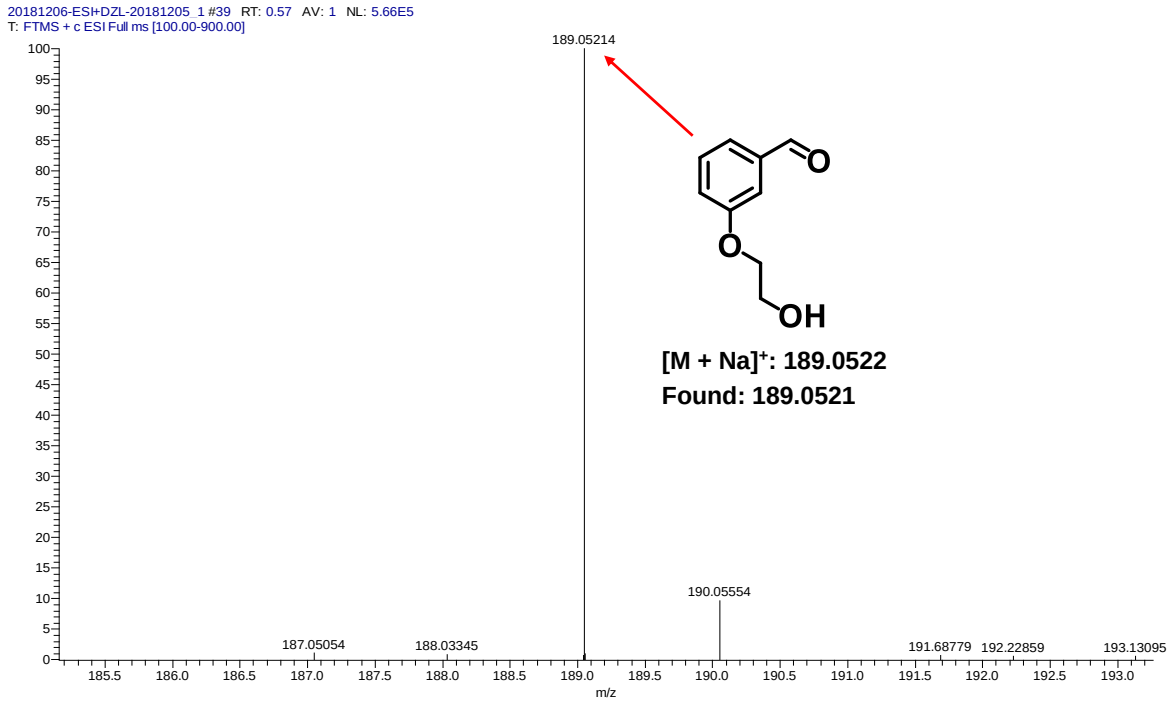

Figure S2. ESI mass spectrum recorded for compound ***1***.

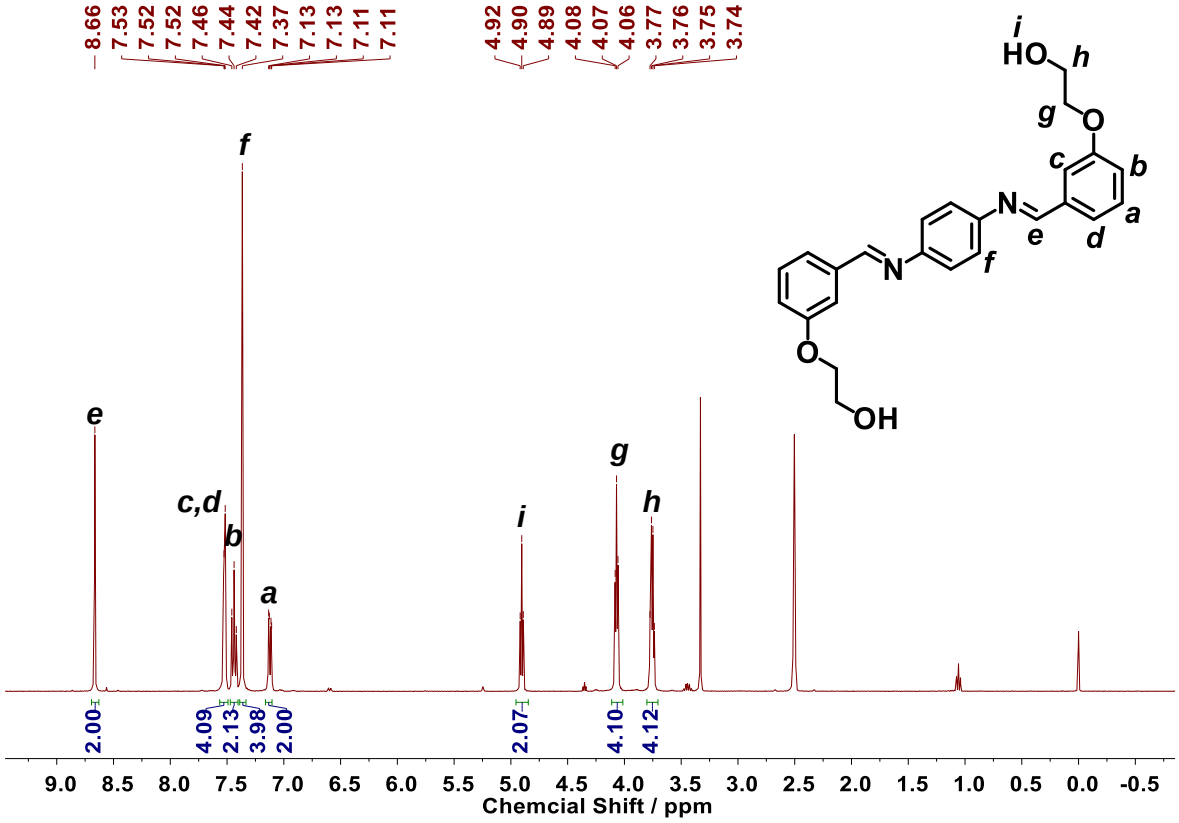

Figure S3. ^{1}H NMR spectrum recorded in DMSO-d_6 for compound ***2***.

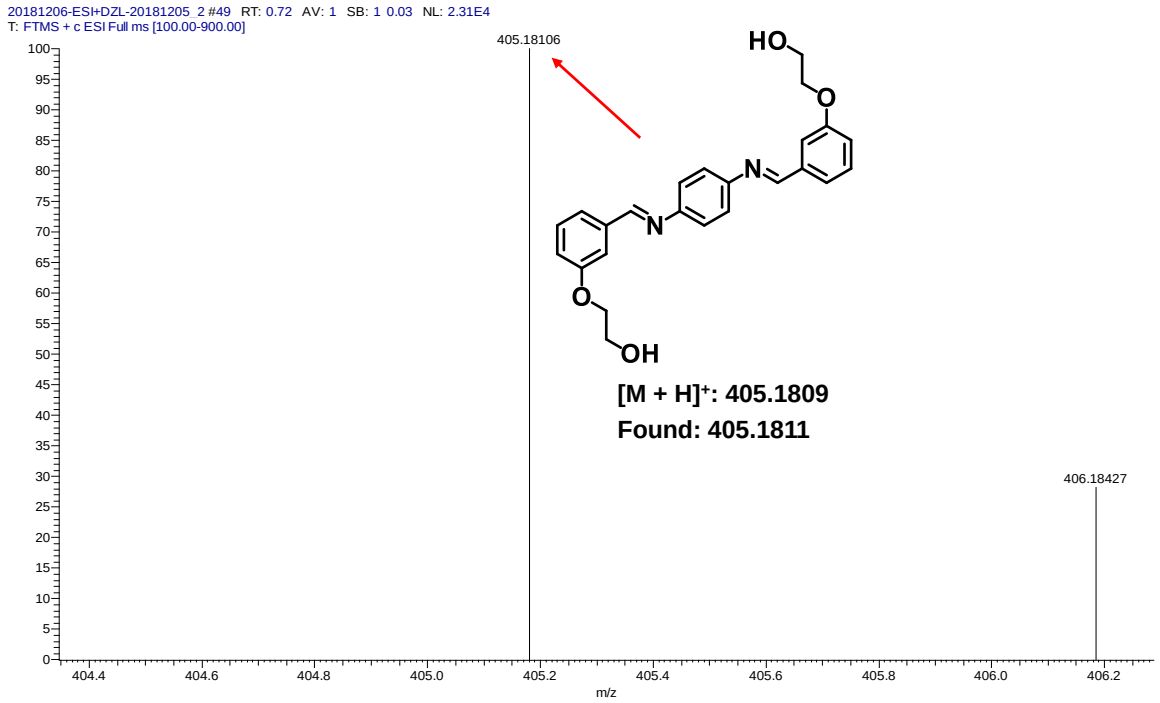

Figure S4. ESI mass spectrum recorded for compound ***2***.

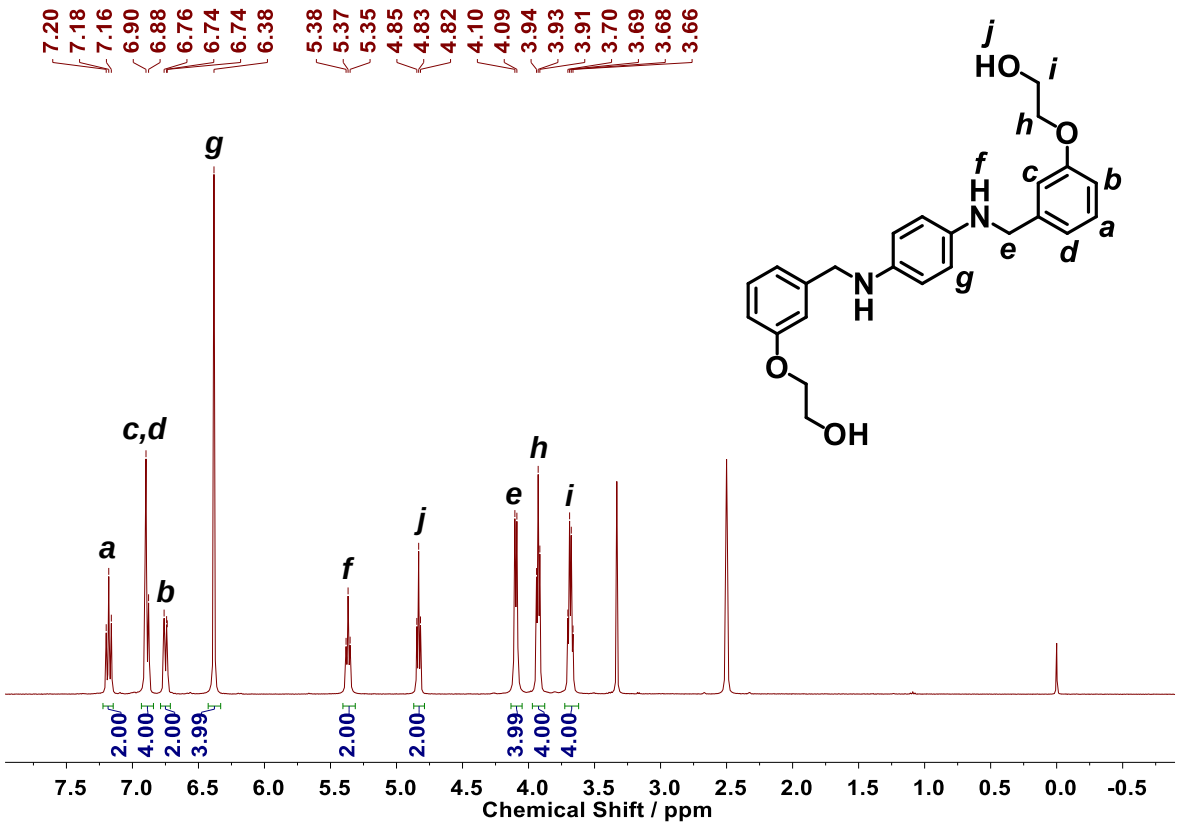

Figure S5. ^{1}H NMR spectrum recorded in DMSO-d_6 for compound ***3***.

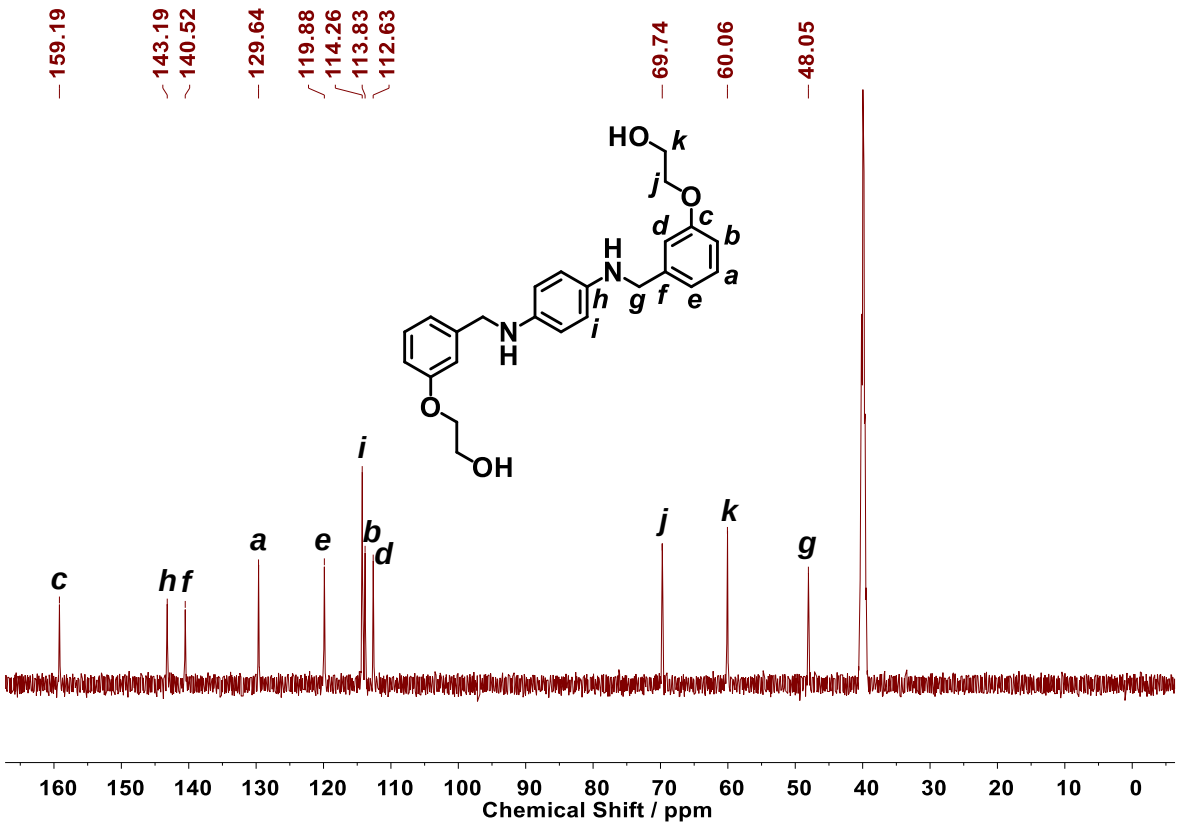

Figure S6. ^{13}C NMR spectrum recorded in DMSO-d_6 for compound ***3***.

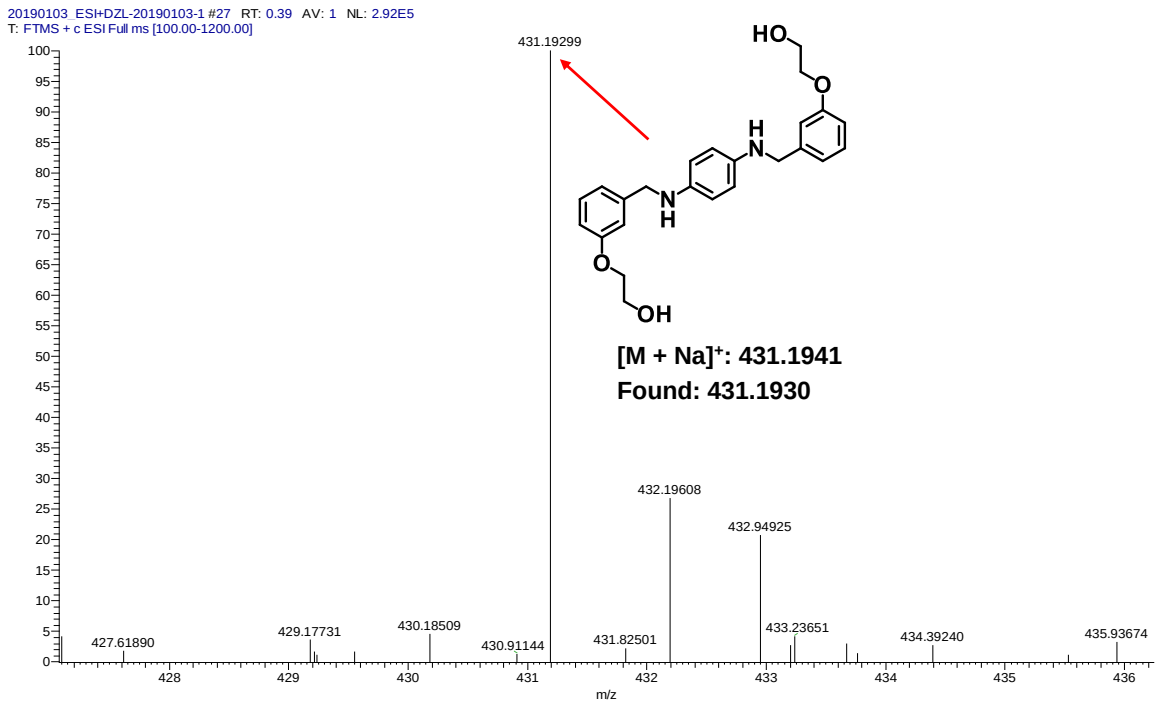

Figure S7. ESI mass spectrum recorded for compound ***3***.

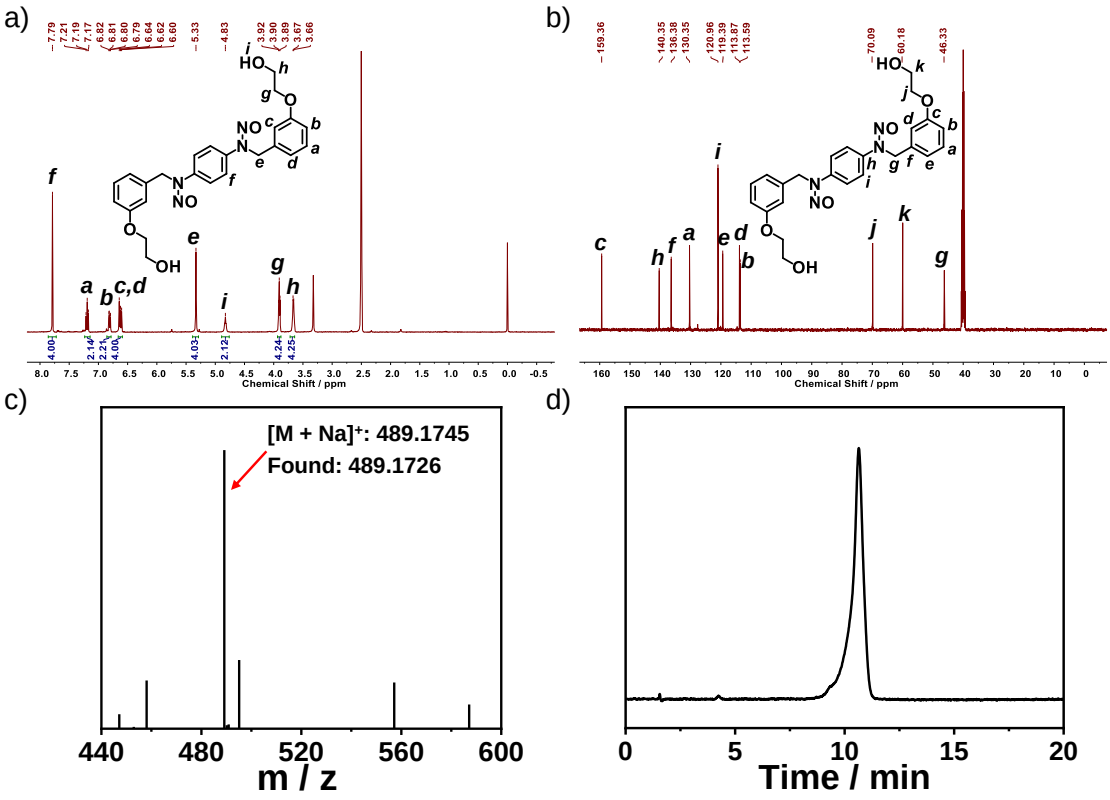

Figure S8. (a) ^{1}H and (b) ^{13}C NMR spectra recorded in DMSO-d_6 for compound ***4***. (c) ESI mass spectrum recorded for compound ***4***. (d) HPLC trace recording at 300 nm for compound ***4*** (20 μM; CH_3CN/H_2O, v/v = 4/6 as the eluent).

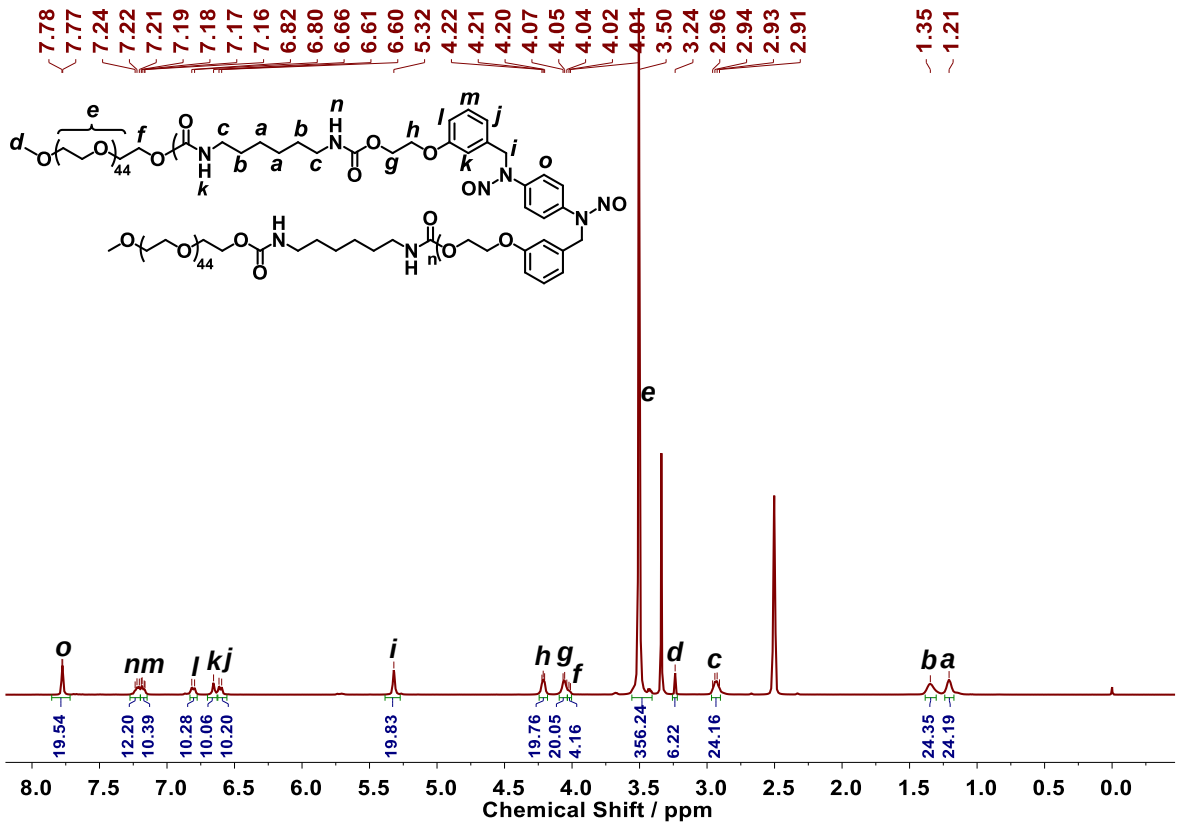

Figure S9. ^{1}H NMR spectrum recorded in DMSO-d_6 for PEG$_{45}$-*b*-PNORM-*b*- PEG$_{45}$ (***5***) triblock copolymer.

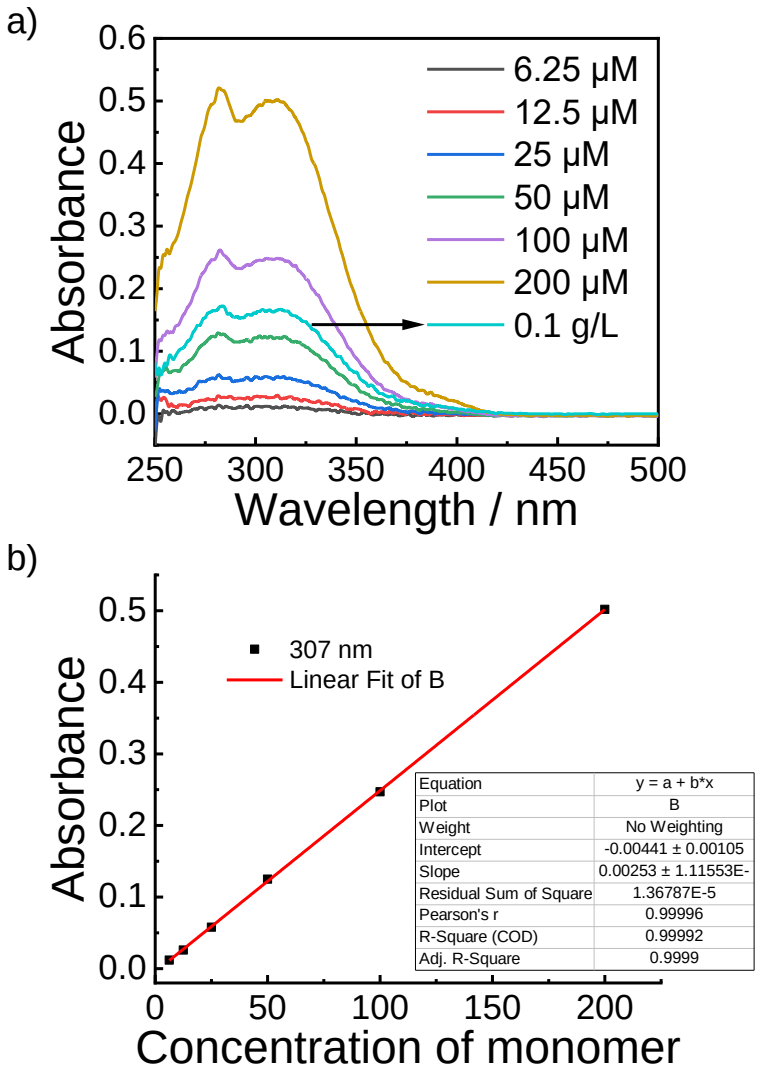

Figure S10. (a) Absorbance spectra of varying concentrations of compound ***4*** and 0.1 g/L of polymer ***5*** in DMSO. (b) Calibration curve fitted by the absorbance intensities at 307 nm against the concentrations of compound ***4***.

Note: The NORM concentration within the PEG_{45}-*b*-$PNORM_5$-*b*-PEG_{45} triblock copolymer at a concentration of 0.1 g/L was determined to be 68.9 μM according to the calibration curve in Figure S10b. From the NMR result shown in Figure S9, the NORM concentration at the same polymer was calculated to be 68 μM, in good agreement with the UV-Vis result, which suggested a complete functionalization of the chain terminals with PEG residues. As such, the as-prepared triblock copolymer was denoted as PEG_{45}-*b*-$PNORM_5$-*b*-PEG_{45}.

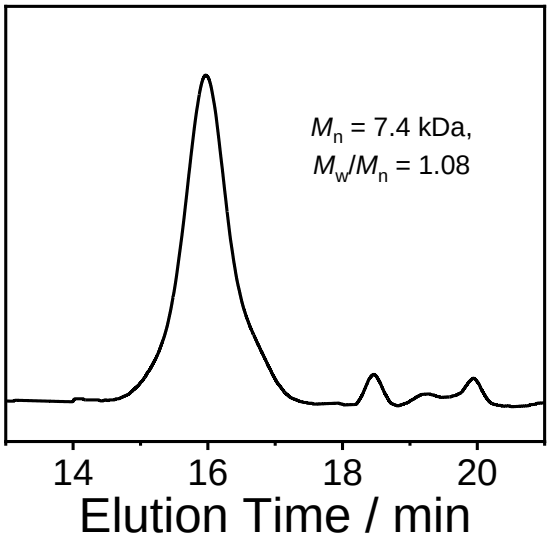

Figure S11. GPC trace of PEG_{45}-*b*-$PNORM_5$-*b*-PEG_{45} triblock copolymer.

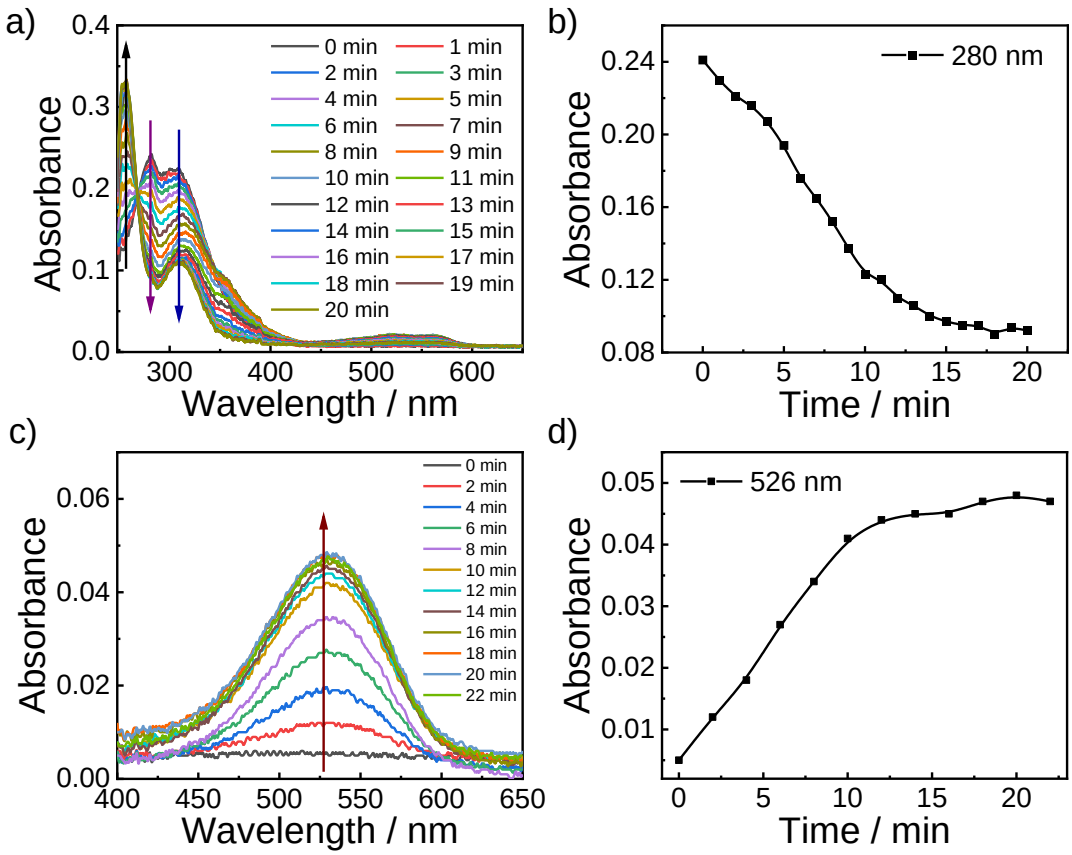

Figure S12. (a) Absorbance spectra and (b) absorbance intensity changes of compound ***4*** (20 μM; DMSO/H_2O =1/1, v/v) under 410 nm irradiation (9.5 mW/cm^2). (c) Absorbance spectra and (d) absorbance intensity changes at 526 nm of compound ***4*** (20 μM; DMSO/H_2O =1/1, v/v) upon irradiation, followed by incubation at room temperature for 10 min with an equivalent volume of Griess reagent.

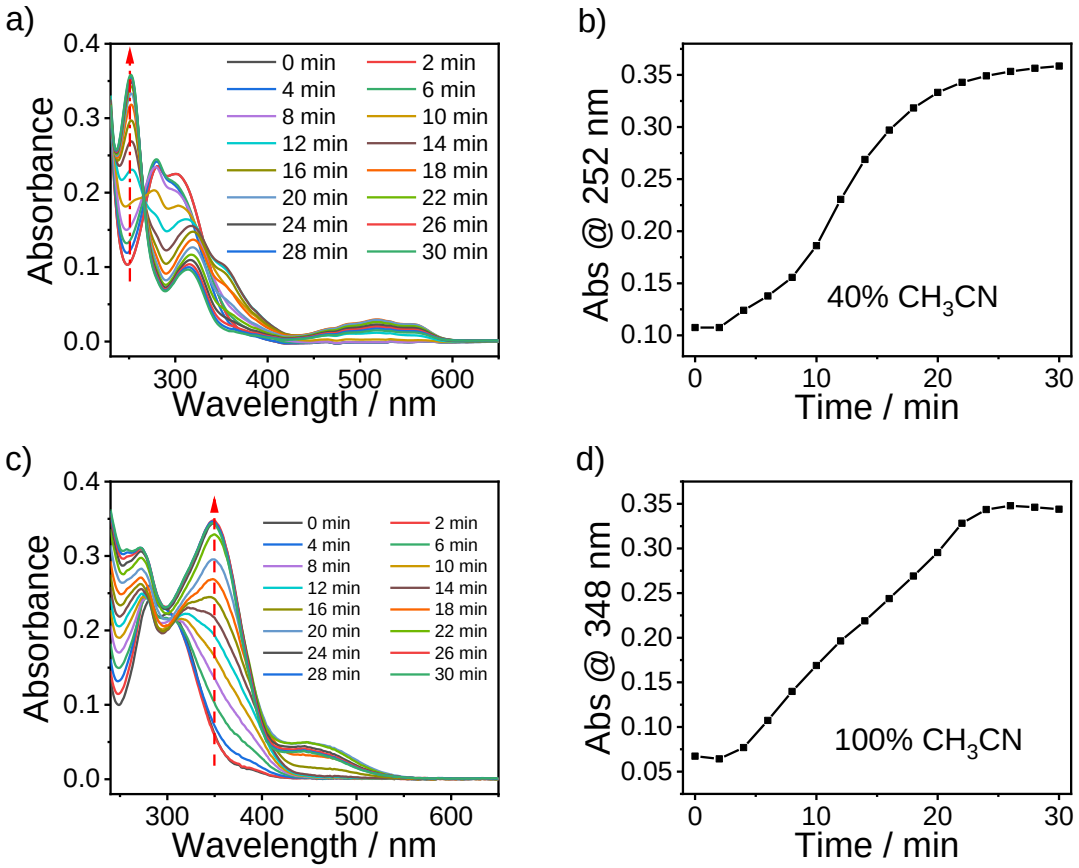

Figure S13. (a,c) Absorbance spectra and (b,d) absorbance intensity changes of compound ***4*** in (a,b) MeCN/H_2O (4/6, v/v) and (c,d) pure MeCN, respectively.

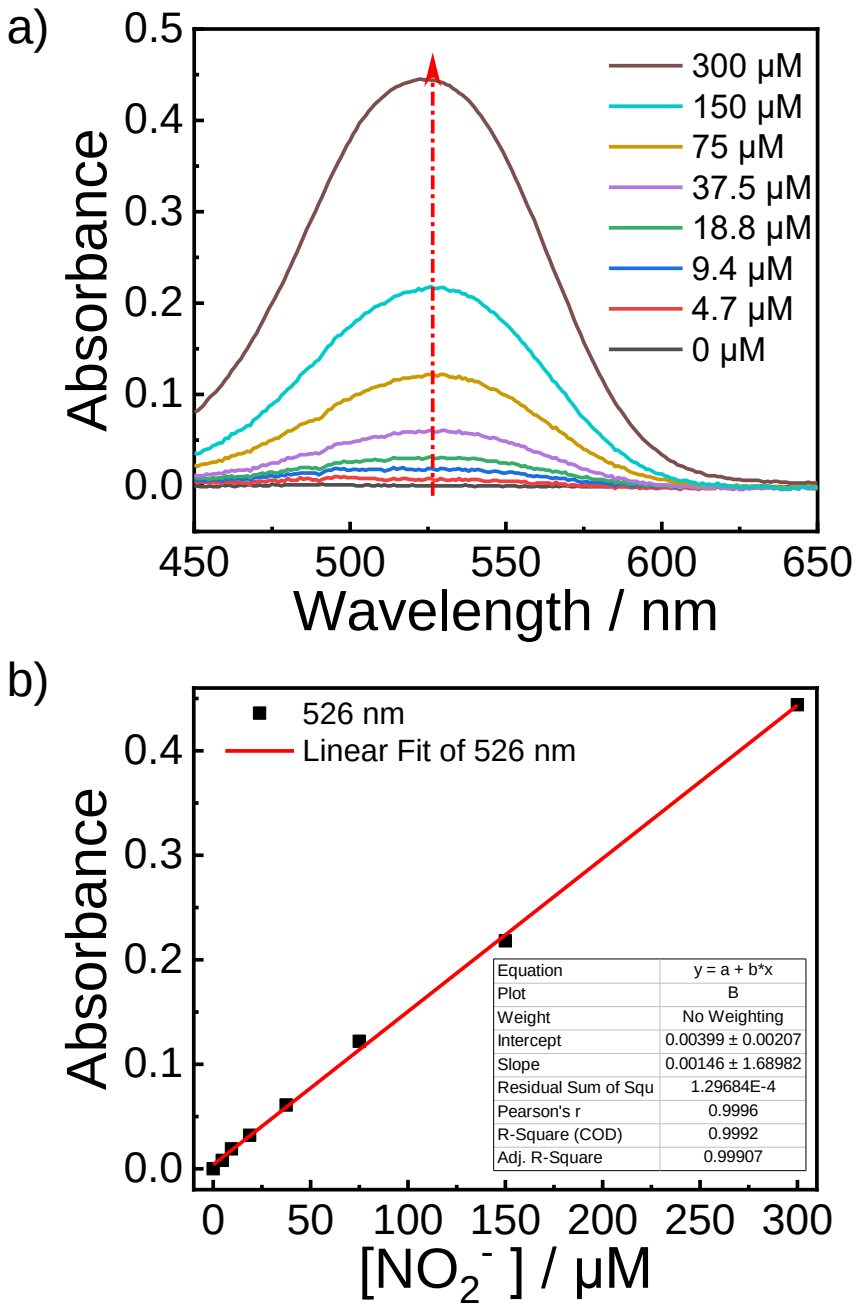

Figure S14. (a) Absorbance spectra of Griess reagent upon addition of varying concentration of nitrite, followed by incubation at room temperature for 10 min under dark and (b) absorbance intensities (black dots) together with a linear fitting curve (red line) at 526 nm against nitrite concentrations.

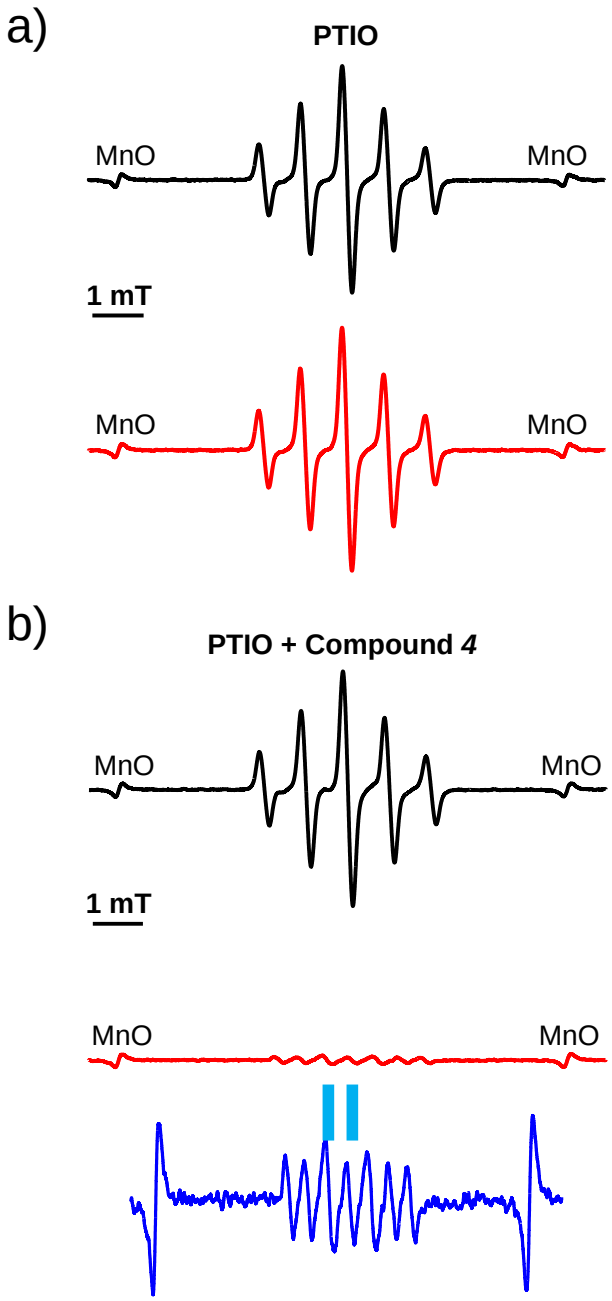

Figure S15. Electronic paramagnetic resonance (EPR) spectra of aqueous solutions (DMSO/H_2O = 2/8, v/v) of (a) PTIO (40 μM) and (b) compound ***4*** (20 μM) with PTIO (40 μM) before (black lines) and after (red lines) irradiation at 410 nm (9.5 mW/cm^2) for 20 min. The blue curve shows the zoomed result of compound ***4*** with PTIO after irradiation for 20 min. Manganese oxide (MnO) was used as an internal standard.

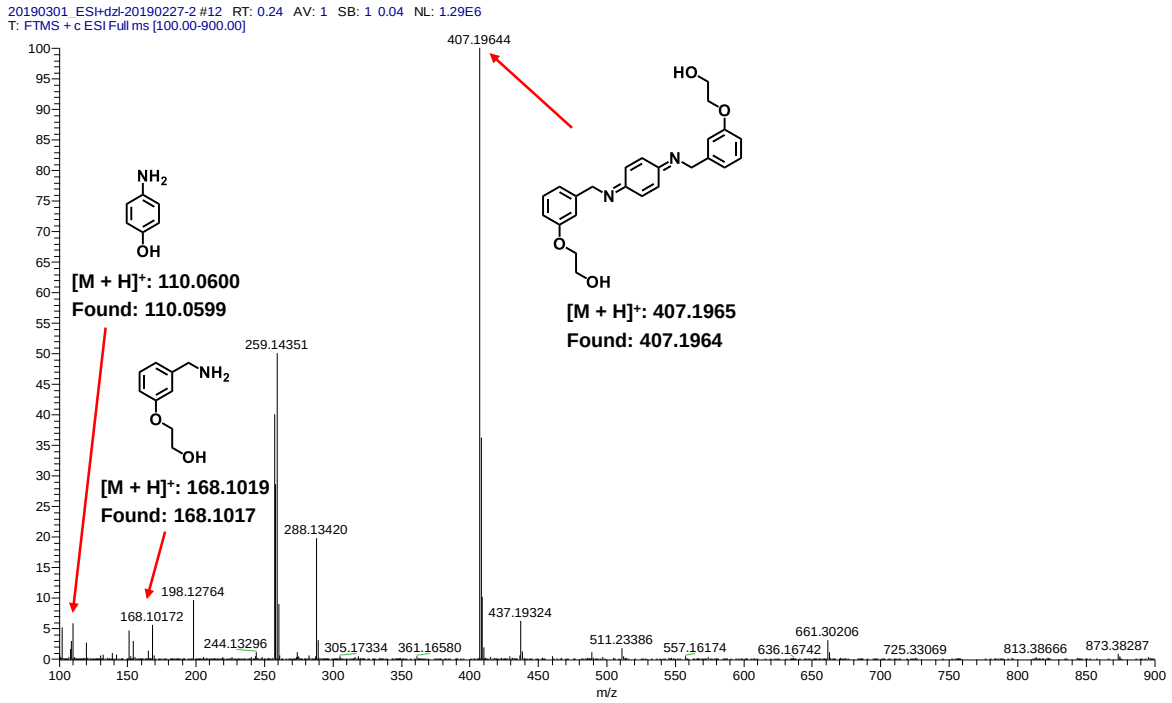

Figure S16. ESI mass spectrum recorded for compound ***4*** under 410 nm irradiation (9.5 mW/cm^2) for 10 min.

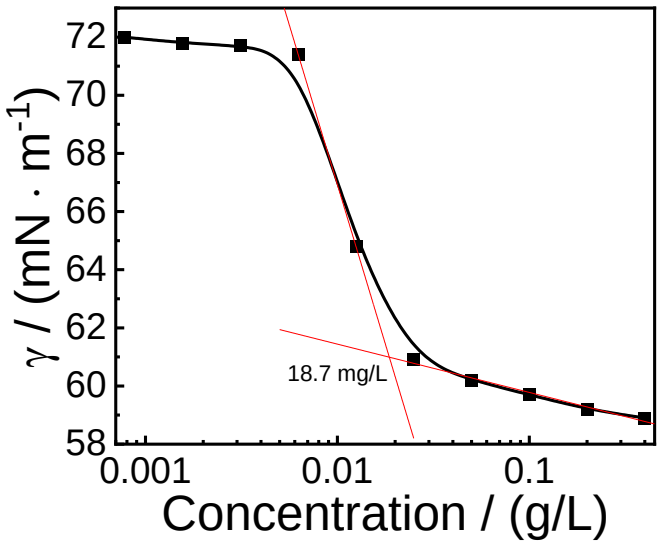

Figure S17. Variation of the surface tension of the aqueous dispersion of PEG_{45}-*b*-$PNORM_5$-*b*-PEG_{45} as a function of the concentration.

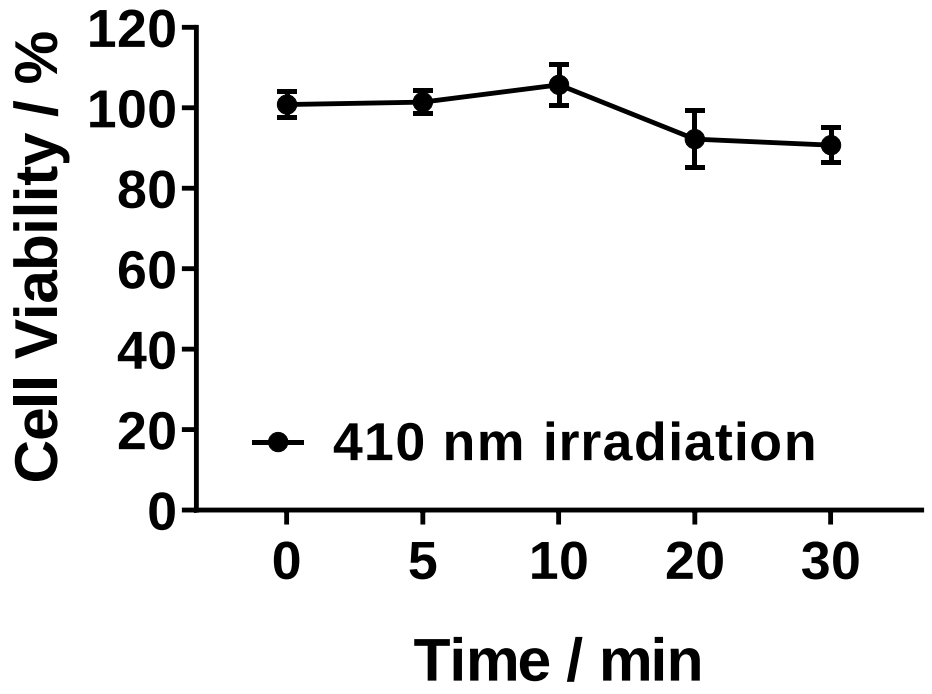

Figure S18. Cytotoxicity of HeLa cells under 410 nm light irradiation (31.5 mW /cm^2).

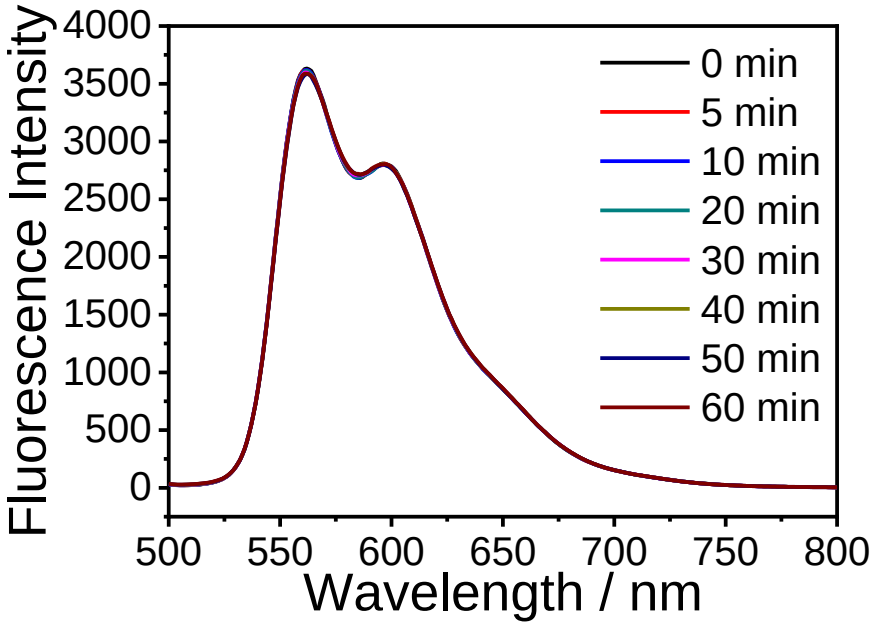

Figure S19. Evolution of fluorescence spectra (λ_{ex} = 480 nm; slit width: Ex. 10 nm, Em. 10 nm) of DMSO solution of DOX (1.75 μg/mL) under 410 nm light irradiation (9.5 mW/cm^2).

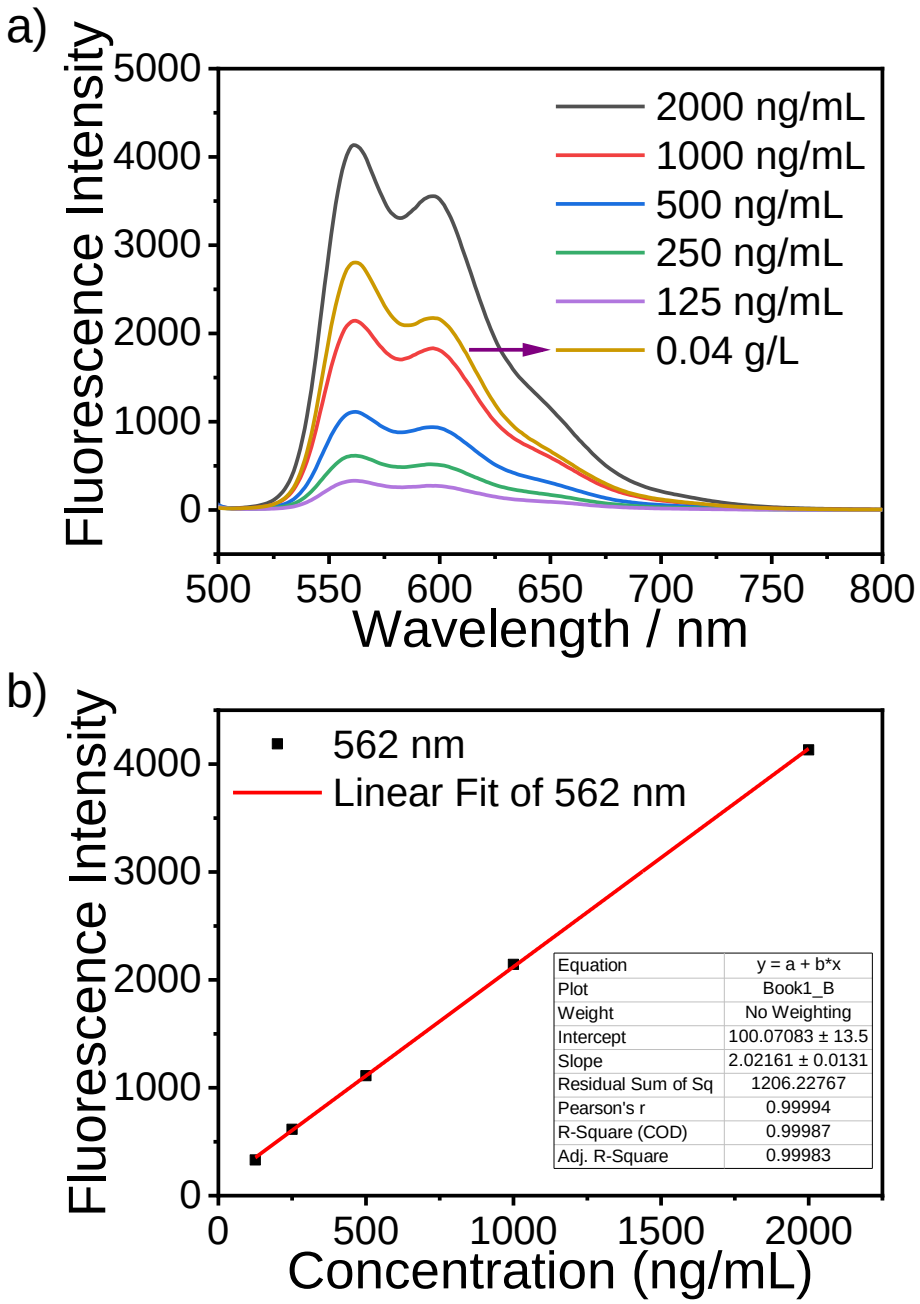

Figure S20. (a) Fluorescence spectra of varying concentrations of DOX and 0.04 g/L of DOX-loaded micelles in DMSO (λ_{ex} = 480 nm; slit width: Ex. 10 nm, Em. 10 nm) and (b) linear fitting of DOX fluorescence intensity against concentration.

Note: The DOX concentration within the micelles at a micelle concentration of 0.04 g/L was determined to be 1.34 μg/mL according to the calibration curve in Figure S20b. The drug loading content (DLC) and the drug loading efficiency (DLE) were thus calculated to be 3.3% and 13.4%, respectively.

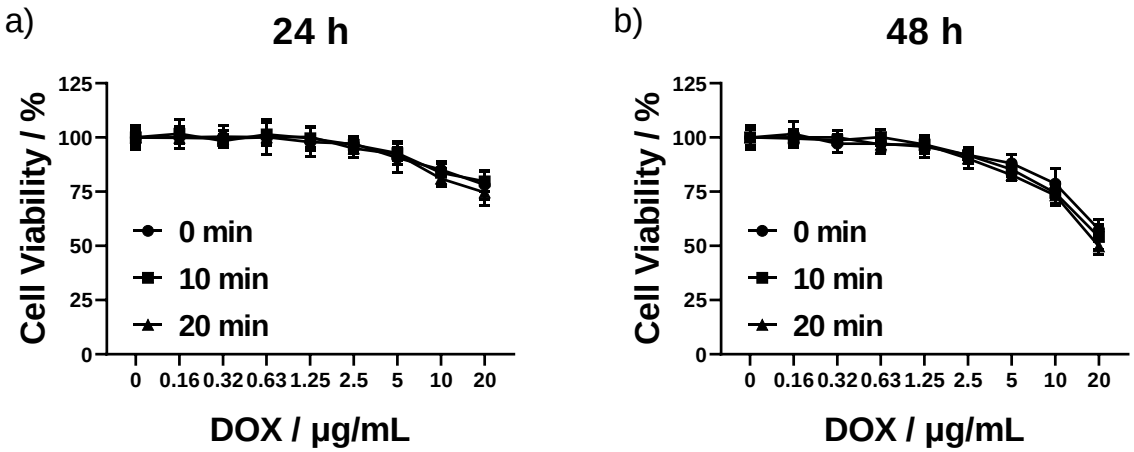

Figure S21. Cytotoxicity assay of MCF-7/ADR cells against DOX after incubation for (a) 24 h and (b) 48 h with varying irradiation durations (0, 10, and 20 min).

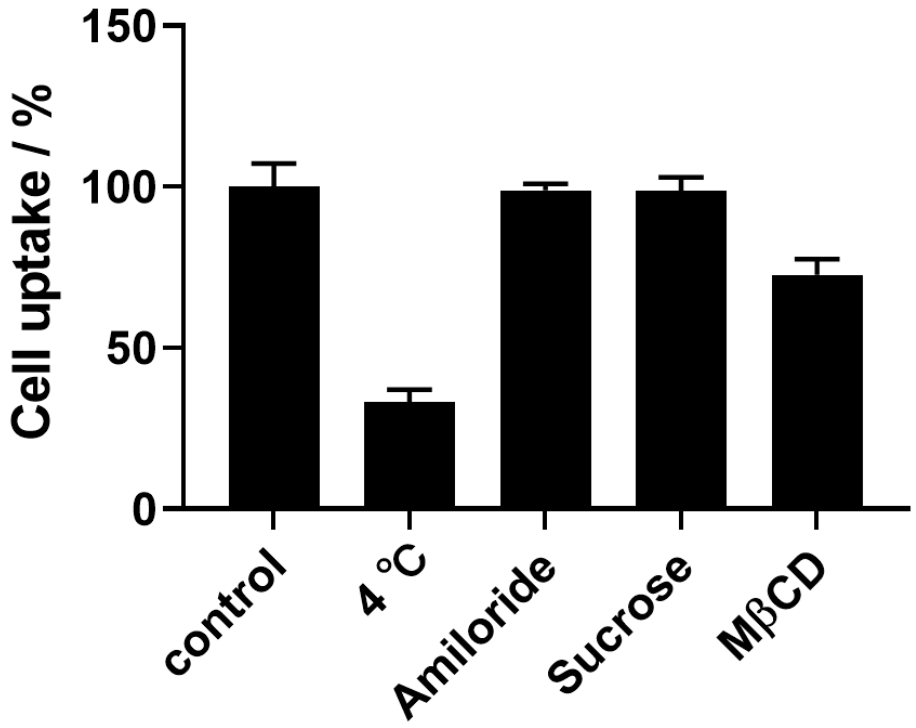

Figure S22. Cellular uptake of NO-releasing PEG_{45}-*b*-$PNORM_5$-*b*-PEG_{45} micelles in MCF-7/ADR cells after 6 h incubation at varying conditions.