Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information

Visible Light-Responsive Micelles Enables Co-delivery of Nitric Oxide and Antibiotics for Synergistic Antibiofilm Applications

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Experimental Section

Materials

Sodium nitrite ($NaNO_2$), tetrabutylammonium bromide (TBAB), potassium carbonate (K_2CO_3), sodium borohydride ($NaBH_4$), sodium sulfate anhydrous (Na_2SO_4), and dibutyltin dilaurate (DBTL) were purchased from Sinopharm Co., Ltd. 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), 2-bromo-1,1,2-triphenylethylene, 4-formylphenylboronic acid, and ciprofloxacin (Cip) were purchased from Energy Chemical. Griess Reagent, 2-Isocyanatoethyl methacrylate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. 2,2′-Azobis(2,4-dimethyl-4-methoxyvaleronitrile) (V70) was purified by recrystallization from ethyl acetate and hexane. LIVE/DEAD® BacLight™ bacterial viability kit reagents (L13152, Molecular Probes, Thermo Fisher) were used as received. Water was deionized with a Milli-Q SP reagent water system (Millipore) to a specific resistivity of 18.4 MΩ cm. 4-(1,2,2-Triphenylvinyl)benzaldehyde[1] and PEG_{45}-based macroRAFT agent[2] were prepared according to previous literature reports. All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. and were used as received without otherwise notification. *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 27853) and PAO1 were used for antibiofilm and antimicrobial studies.

Sample Preparation

Synthesis of (4-((4-(1,2,2-triphenylvinyl)benzyl)amino)phenyl)methanol (Compound 3, Scheme 2a). Pd/C (0.15 g) and 4-nitrobenzyl alcohol (1.50 g, 9.8 mmol) were dissolved in CH_3OH (25 mL), and stirred at room temperature in a hydrogen atmosphere for 1.5 h. Then, the reaction mixture was filtered and the filtrate was condensed to obtain 4-aminobenzyl alcohol, which was directly used for the following reactions without further purification. Next, 4-(1,2,2-triphenylvinyl)benzaldehyde (2.8 g, 7.8 mmol) and 4-aminobenzyl alcohol (0.8 g, 6.5 mmol) were dissolved in 50 mL C_2H_5OH and stirred at 85 °C for 14 h. After removing the solvent under vacuum, the residue was washed with cold ethanol. Then, the crude products (1.5 g, 3.22 mmol)

were dissolved in 40 mL THF/MeOH (*v*/*v*, 1/1). The resulting Schiff base was then reduced with $NaBH_4$. Specifically, $NaBH_4$ (0.24 g, 6.44 mmol) was added and stirred at room temperature for 4 h. Then, 10 mL of water was added into the reaction mixture and THF was removed under reduced pressure. The residue was extracted with dichloromethane and the organic phase was dried over anhydrous Na_2SO_4 to obtain compound ***3***. The crude product of compound ***3*** was used for the following reaction without further purification procedure.

Synthesis of N-(4-(hydroxymethyl)phenyl)-N-(4-(1,2,2-triphenylvinyl)benzyl) nitrous amide (Compound 2, Scheme 2a). Compound ***3*** (0.5 g, 1.07 mmol) was dissolved in a mixture of glacial acetic acid (20 mL), and an aqueous solution (15 mL) of sodium nitrite ($NaNO_2$, 0.37 g, 5.35 mmol) was slowly added. Then, the mixture was further stirred at room temperature for 2 h, and poured into a saturated $NaHCO_3$ solution. The aqueous phase was extracted with DCM (50 mL × 3) and the combined organic phase was dried over Na_2SO_4. The residue was purified by column chromatography to obtained compound ***2*** (0.457 g, yield: 86%). ^{1}H NMR (400 MHz, $CDCl_3$, Figure S1a) 7.49 – 7.38 (m, 4H), 7.12 – 7.05 (m, 9H), 7.02 – 6.89 (m, 8H), 6.81 (d, J = 7.8 Hz, 2H), 5.14 (s, 2H), 4.75 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$, Figure S1b) 143.50, 143.35, 143.20, 141.35, 141.09, 140.24, 140.03, 132.29, 131.94, 131.72, 131.26, 129.17, 127.91, 126.53, 119.92, 64.63, 47.09. ESI-MS (Figure S1c): m/z calc. for $C_{34}H_{28}N_2O_2$ $[M + Na]^+$: 519.20430, found: 519.20427.

Synthesis of NO-releasing monomer (Compound 1, Scheme 2a). Compound ***2*** (0.4 g, 0.81 mmol) was dissolved in THF (30 mL), and DBTL (150 μL) was added. After that, 2-isocyanatoethyl methacrylate (187 mg, 1.21 mmol) was added into the mixture. The mixture was stirred at room temperature overnight. After removing the solvent under vacuum, the solid was further purified by flash chromatography obtain NO-releasing monomer of compound ***1*** (0.456 g, yield: 87%). ^{1}H NMR (400 MHz, $CDCl_3$, Figure S2a) 7.49 – 7.38 (m, 4H), 7.11 – 7.03 (m, 9H), 7.02 – 6.89 (m, 8H), 6.80 (d, J = 8.1 Hz, 2H), 6.10 (s, 1H), 5.58 (t, J = 1.6 Hz, 1H), 5.14 (s, 4H), 5.05 (d, J = 6.3 Hz, 1H), 4.25 (t, J = 5.3 Hz, 2H), 3.53 (q, J = 5.6 Hz, 2H), 1.93 (t, J = 1.3 Hz, 3H). ^{13}C

NMR (101 MHz, $CDCl_3$, Figure S2b) 167.29, 156.15, 143.50, 143.48, 143.33, 143.23, 141.57, 141.37, 140.22, 135.91, 135.55, 132.24, 131.75, 131.27, 129.25, 127.70, 127.65, 126.52, 126.50, 126.45, 126.14, 119.61, 66.12, 63.68, 46.92, 40.35, 18.33. HPLC analysis (Figure S2c): elution peak at 6.4 min (8/2, v/v, MeCN/H_2O). ESI-MS (Figure S2d): m/z calc. for $C_{41}H_{37}N_3O_5$ $[M + Na]^+$: 674.26254, found: 674.26318.

Synthesis of PTNO amphiphilic block copolymer (Scheme 2a). Monomer ***1*** (0.2 g, 0.3 mmol, 30 eq.), V70 (0.63 mg, 0.002 mmol, 0.2 eq.), and PEO-based macroRAFT agent (23 mg, 0.01 mmol, 1 eq.) were dissolved in DMSO (0.4 mL) and were charged into a reaction tube equipped with a magnetic stirring bar. The reaction tube was carefully degassed by three freeze-pump-thaw cycles and was sealed under vacuum. After stirring at 30 ^oC for 15 h, the polymerization reaction was quenched by immersing the reaction tube into liquid nitrogen and diluted with THF. The THF solution was then precipitated into an excess amount of diethyl ether to obtain the PTNO diblock copolymer. The above dissolution-precipitation cycle was repeated twice. The final product was dried in a vacuum oven at room temperature to obtain PTNO diblock copolymers as a yellowish solid. The degree of polymerization, DP, of the NO-releasing block, was determined to be 18 or 13 according to ^1H NMR analysis in $CDCl_3$ (Figure S3). GPC analysis of PTNO1 and PTNO 2 revealed an M_n of 9.6 and 8.9 kDa and an M_w/M_n of 1.13 and 1.14, respectively (Figure S4, Table S1).

Self-Assembly of PTNO1 block copolymer. PTNO1 diblock copolymer (1 mg) was dissolved in 1 mL of DMF, which was quickly injected into 8 mL of DI water in one shot. After that, DMF was removed by dialyzed (MWCO = 14 kDa) against DI water. The resultant micellar nanoparticles were stored at 4 ^oC in a refrigerator and used for further experiments.

Fabrication of Cip-Loaded Micelles (Cip@PTNO1). PTNO1 (2 mg) and ciprofloxacin (Cip) (0.1 mg) were dissolved in DMF (1 mL) and the DMF solution was quickly injected into DI water (8 mL). DMF was removed by dialyzed against DI water (MWCO = 14 kDa). To quantify the drug-loading content (DLC) and the drug-loading efficiency (DLE) of Cip in Cip@PTNO1 micelles, the micelles were centrifuged by

ultrafiltration (10000 Da, 8000 rpm, 30 min). After that, the filtrate was lyophilized and dissolved in an aqueous solution (pH 2.0), and the absorption wavelength of Cip at 277 nm was recorded (Figure S12). The drug-loading content (DLC) and drug loading efficiency (DLE) of Cip in the Cip@PTNO1 micelles were calculated to be 3.1% and 62%, respectively. Therefore, the loaded Cip concentration was calculated to be ~6.2 μg/mL at the Cip@PTNO1 micelles concentration of 0.2 g/L.

Visible Light-Mediated Cip Release. Cip@PTNO1 micelles (0.2 mg/mL; 0.8 mL) were placed in sample vials (30 mL) and irradiated with a 410 nm LED lamp (28 mW/cm^2) for 30 min. The same micellar dispersion without light irradiation was used as control. The three micellar dispersions were dialyzed against acetic acid buffer (8 mL, pH 5.5) at 37 ^oC under constant stirring. At predetermined time points, the dialysates were collected and replaced with 8 mL of fresh acetic acid buffer solution. The dialysates were subjected to lyophilization and the residues were dissolved in an aqueous solution (pH 2.0). The Cip releasing contents were calculated against a standard calibration curve of Cip at 277 nm established in a purely aqueous solution (pH 2.0).

General Procedures for Bacterial Biofilm Formation and Harvesting. *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 27853) were used for antibiofilm experiments. *P. aeruginosa* suspension (10^8 CFU/mL, 100 μL) and Tryptic soy broth (TSB) medium (400 μL) were added into each well in a 24-well plate. The plate was placed in an incubator and cultured at 37 °C. After 24 h, the timeworn medium was replaced with fresh TSB medium. After incubation for another 24 h, the biofilms were used for further antibiofilm experiments.

Visible Light-Mediated NO Release for Biofilm Dispersal. Preformed *P. aeruginosa* biofilms were treated for 30 min (37 ^oC, 120 rpm) with free Cip (3 μg/mL), PTNO1 (0.2 mg/mL), and Cip@PTNO1 (0.2 mg/mL), respectively. Then, the biofilms were irradiated or not irradiated with an LED lamp for 30 min (410 nm, 28 mW/cm^2). After that, the biofilms receiving different treatments were further incubated for 1 h before antibiofilm evaluation.

Quantification of the Biofilm Biomass by Crystal Violet Staining. Biofilm biomass was quantified by the crystal violet (CV) staining. Briefly, after various treatments, the OD_{600} values of the culture supernatant were measured using a microtiter plate reader (Thermo Fisher). The residual biofilms together with the biofilms adhered to the well of the culture plates were washed two times with PBS (1 mL), followed by the addition of the crystal violet staining agent (0.5 mL, 0.1 wt% in PBS). Next, the plate was incubated for 20 min before the wells were washed with PBS two times. The crystal violet was dissolved in 500 μL of pure ethanol and was quantified by measuring the OD_{550} of the homogenized suspension using a microtiter plate reader (Thermo Fisher). All the reported results were repeated in at least three independent experiments.

Evaluation of the Bacterial Viability by Colony Forming Unit (CFU) Assay. The bacterial viability in biofilms was evaluated by a CFU assay. After various treatments, the planktonic and biofilms were evaluated by a drop plate method. Briefly, for planktonic analysis, the supernatants were serially diluted with PBS and were plated onto LB agar. For biofilms, the residual biofilms were washed twice with PBS (500 μL); the cells were homogenized by ultrasound (200 W, 40 kHz) for 15 min and resuspended in PBS. Then, the supernatants were serially diluted with PBS and plated onto LB agar. After 24 h incubation at 37 ^{o}C, the planktonic and biofilm colonies were counted. All assays included three replicates and were repeated in three independent experiments.

In situ observation of NO release from the micellar nanoparticles within PAO1 Biofilms by confocal laser scanning microscopy (CLSM). PAO1 biofilms were cultured according to the method detailed above, followed by treating with free Cip (3 μg/mL), PTNO1 (0.2 mg/mL), and Cip@PTNO1 (0.2 mg/mL). After 30 min incubation, the biofilms were subjected to irradiation with or without 410 nm for 30 min. Finally, the biofilm was imaged by CLSM. The blue channel recorded the fluorescence of TPE derivatives after NO release was excited at 405 nm and collected at 420-460 nm. The green channel of PAO1 bacteria was excited at 488 nm and collected at 520-

580 nm.

Live/Dead Staining of Biofilm Observed by CLSM. Briefly, *P. aeruginosa* (ATCC 27853) biofilms were grown in a glass-bottom 24-well plate as described above. After various treatments, the biofilms were washed twice with PBS and stained with LIVE/DEAD® BacLight™ bacterial viability kit reagents (L-13152, Molecular Probes) according to the manufacturer's instructions. The biofilms were incubated for 20 min at room temperature under dark conditions and then observed by CLSM. The green channel of SYTO9 was excited at 488 nm and was collected between 500-545 nm; the red channel of PI was excited at 514 nm and collected between 620-670 nm.

In vitro Cytotoxicity Against L929 Cells. The cell viability was determined by a standard MTT assay. Briefly, L929 cells were cultured in DMEM medium with 10% fetal bovine serum (FBS) and antibiotics (0.1 mg/mL streptomycin and 100 units/mL penicillin) at 37 ^{o}C in a humidified atmosphere with 5% CO_2. L929 cells were seeded on a 96-well plate at a density of 10,000 cells/well in 100 μL of DMEM complete medium. After incubation at 37 ^{o}C for 24 h, DMEM medium was replaced by 100 μL of fresh medium containing PTNO1 micelles (0, 0.025, 0.05, 0.1, 0.2 g/L). After incubation for 24 h, 10 μL of MTT reagent (5 mg/mL in PBS buffer) was added to each well, and the cells were further incubated at 37 ^{o}C for 4 h. The culture medium in each well was then removed and 100 μL of DMSO was added. The absorbance values were recorded at the wavelength of 490 nm by a microplate reader (Thermo Fisher). The cell viability was calculated using the following equation:

$$\text{Cell viability\%} = (A_{490,\ \text{sample}} - A_{490,\ \text{blank}})/(A_{490,\ \text{control}} - A_{490,\ \text{blank}}) \times 100\%$$

where $A_{490,\ \text{sample}}$ and $A_{490,\ \text{control}}$ are the absorbance intensities in the presence and absence of PTNO1 micelles. $A_{490,\ \text{blank}}$ is the absorbance value of the culture plate with an identical volume of MTT solution without cells, respectively.

Characterization

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV400 NMR (400 MHz) spectrometer operated in the Fourier transform mode. Deuterated

chloroform ($CDCl_3$) was used as the solvent. UV-Vis absorption spectra were acquired on a TU-1910 double beam UV-Vis spectrophotometer (Puxi General Instrumental Company, China). High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) spectra were performed on Waters XEVO®G2-XS-TOF 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. Electron paramagnetic resonance (EPR) spectra were recorded on a JEOL JES FA200 ESR spectrometer (300 K, 9.063 GHz, Xband) at room temperature. Molecular weights and molecular weight distributions were determined by gel permeation chromatography (GPC) equipped with Waters 1515 pump and Waters 2414 differential refractive index detector (set at 30 °C). It used a series of two linear Styragel columns (HR2 and HR4) at an oven temperature of 35 °C. THF was used as the eluent at a flow rate of 1.0 mL/min. Polystyrene standards with low polydispersities were employed for calibration. Dynamic light scattering (DLS) was conducted on a Zetasizer Nano ZS (Malvern). Transmission electron microscopy (TEM) observations were performed on a JEM-2100 electron microscope at an acceleration voltage of 200 kV. Confocal laser scanning microscopy (CLSM) images were acquired using a Leica TCS SP5 microscope.

References

1. C. Li, J. Zhang, S. Zhang and Y. Zhao, *Angew. Chem., Int. Ed.*, 2019, **58**, 1643-1647.
2. J. Rieger, F. Stoffelbach, C. Bui, D. Alaimo, C. Jerome and B. Charleux, *Macromolecules*, 2008, **41**, 4065-4068.

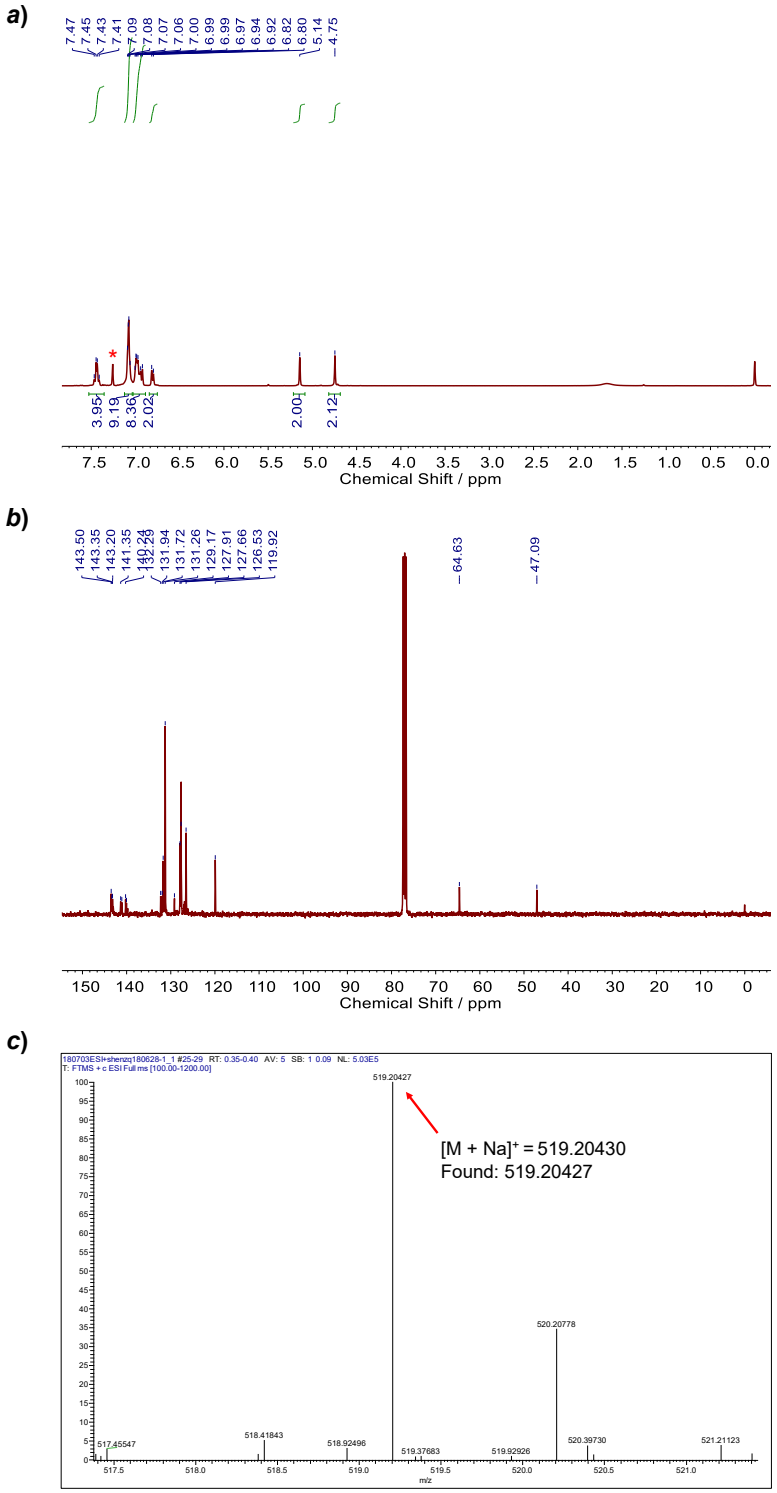

Figure S1. (a) ^{1}H and (b) ^{13}C NMR spectra in $CDCl_3$ and (c) ESI mass spectrum recorded for compound ***2***, respectively.

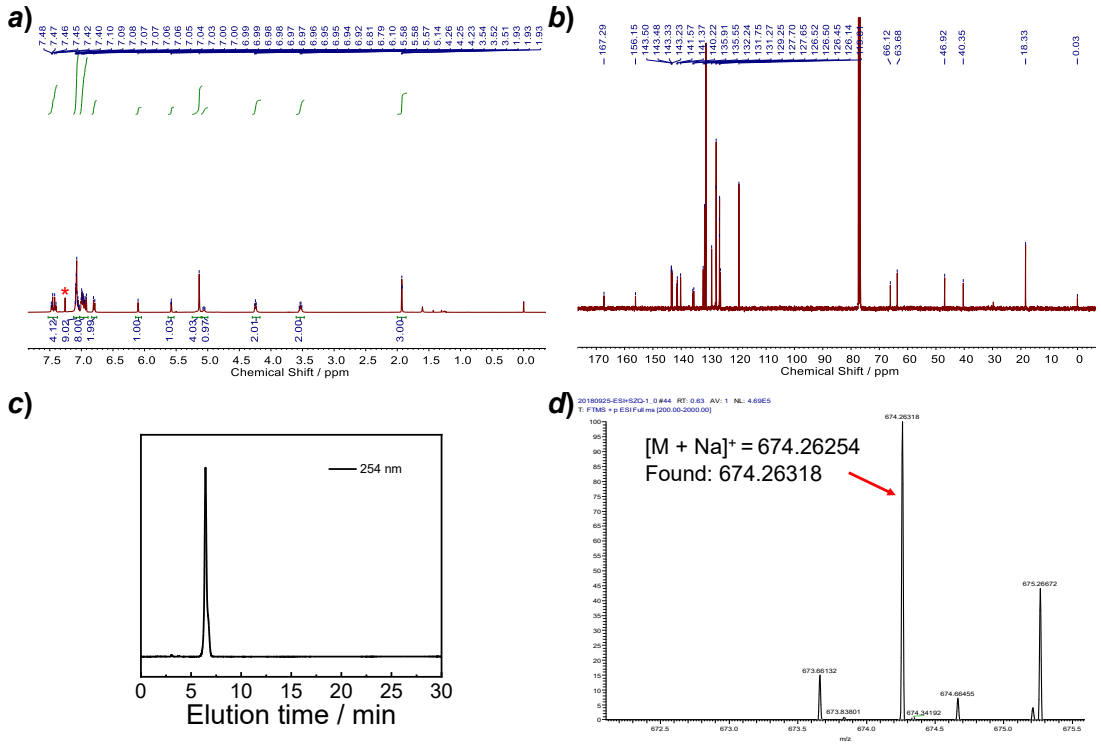

Figure S2. (a) ^{1}H and (b) ^{13}C NMR spectra recorded for compound ***1*** in $CDCl_3$. (c) HPLC elution profile recorded at 254 nm for compound ***1*** using MeCN/H_2O = 8/2 (v/v) as the eluent. (d) ESI mass spectrum recorded for compound ***1***.

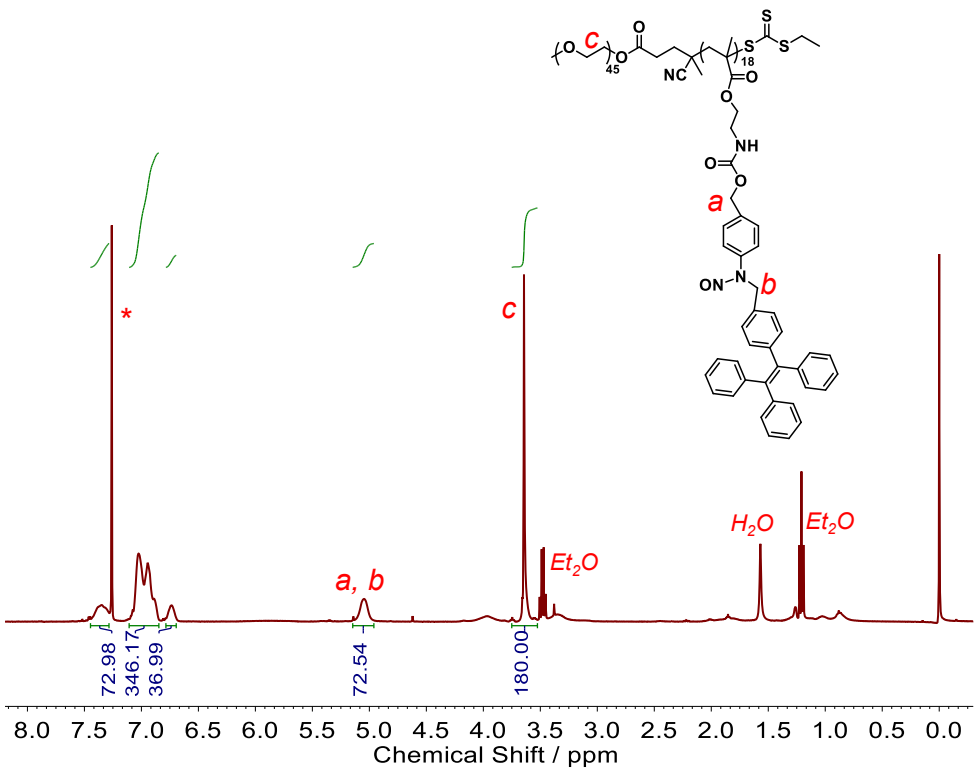

Figure S3. ^{1}H NMR spectra recorded in $CDCl_3$ for PTNO1 diblock copolymer.

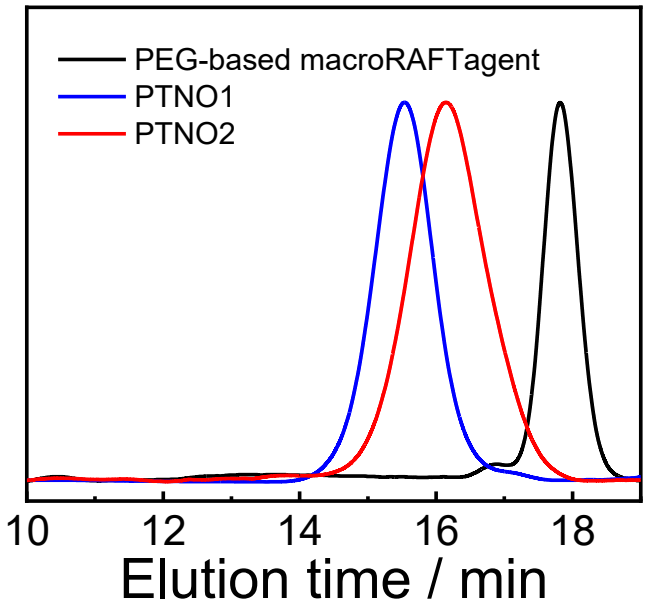

Figure S4. GPC elution profile of PEG-based macroRAFT agent and PTNO block copolymers (**PTNO1 and PTNO2)**.

Table S1. Structural Parameters of the Amphiphilic Block Copolymers Used in This Study.

Entry	Sample[a]	$M_{n,\ NMR}$ (kDa) [a]	$M_{n,\ GPC}$ (kDa) [b]	M_w/M_n [b]	NO Payload [c] (μM)
PTNO1	PEG_{45}-*b*-$PTNO_{18}$	14	9.6	1.13	128.8
PTNO2	PEG_{45}-*b*-$PTNO_{13}$	10.7	8.9	1.14	121.3

[a] calculated from ^{1}H NMR results; [b] determined by GPC using THF as the eluent; [c] theoretical NO loading contents calculated from the ^{1}H NMR results at micellar concentrations of 0.1 g/L.

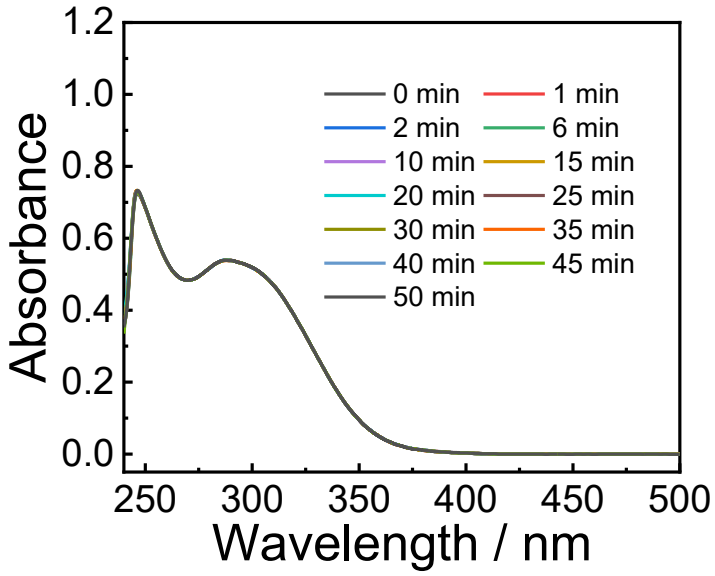

Figure S5. UV-vis spectra of aqueous solutions (DMSO/H_2O = 6/4, v/v) of compound ***1*** without 410 nm irradiation.

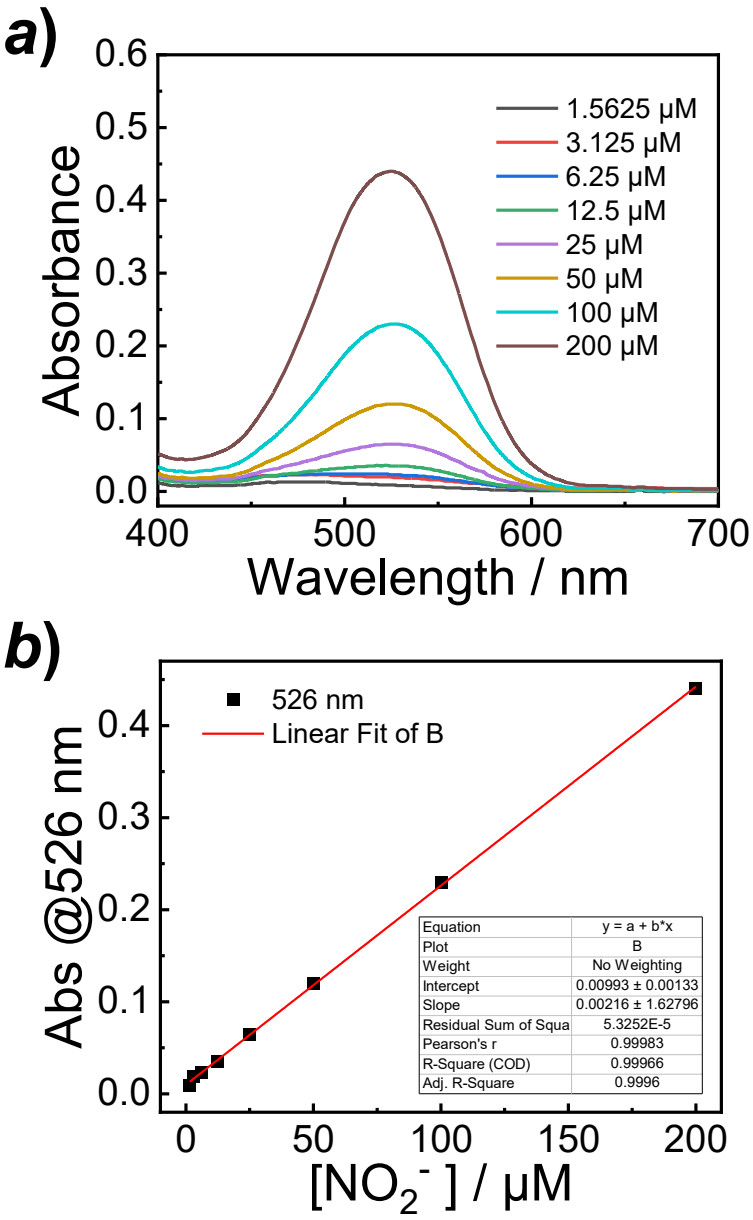

Figure S6. (a) Absorbance spectra of the mixtures of $NaNO_2$ and Griess reagent. (b) Absorbance intensities at 526 nm as a function of nitrite concentrations.

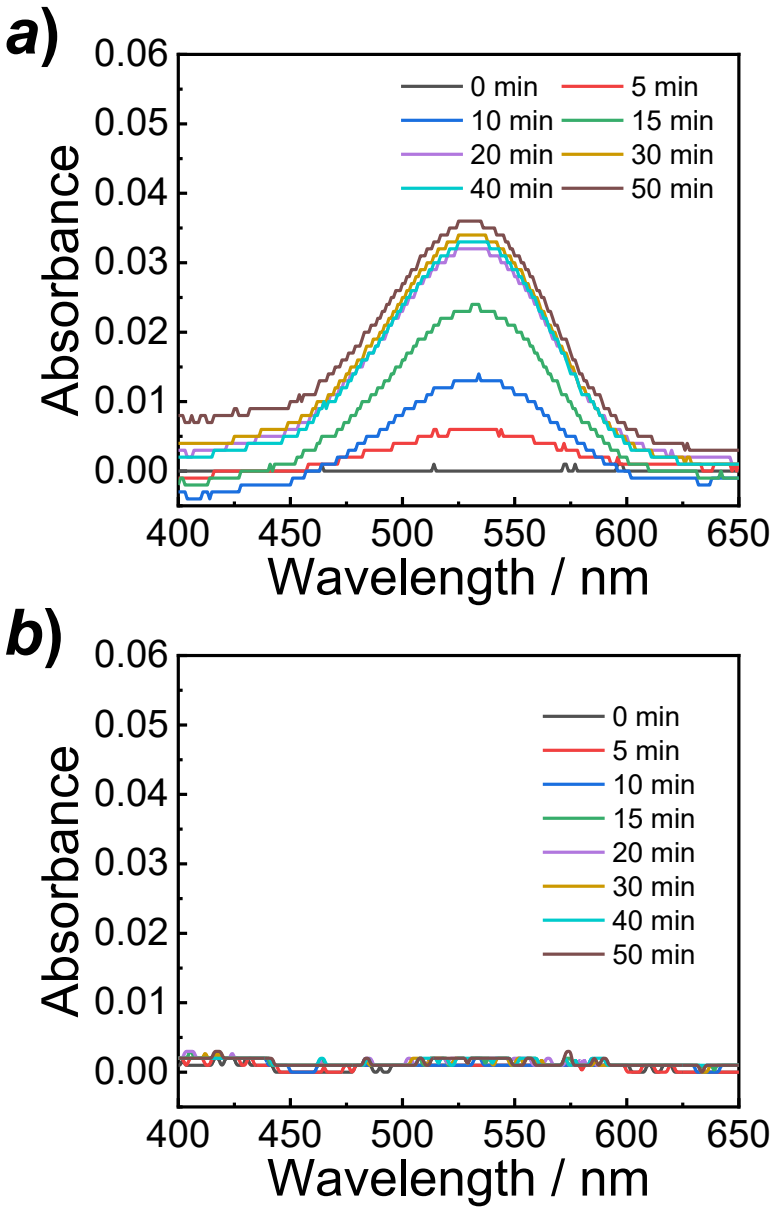

Figure S7. Absorbance spectra of aqueous solutions (DMSO/H_2O = 6/4, v/v) of compound ***1*** (a) with or (b) without 410 nm light irradiation (28 mW/cm^2) as assayed by Griess reagent.

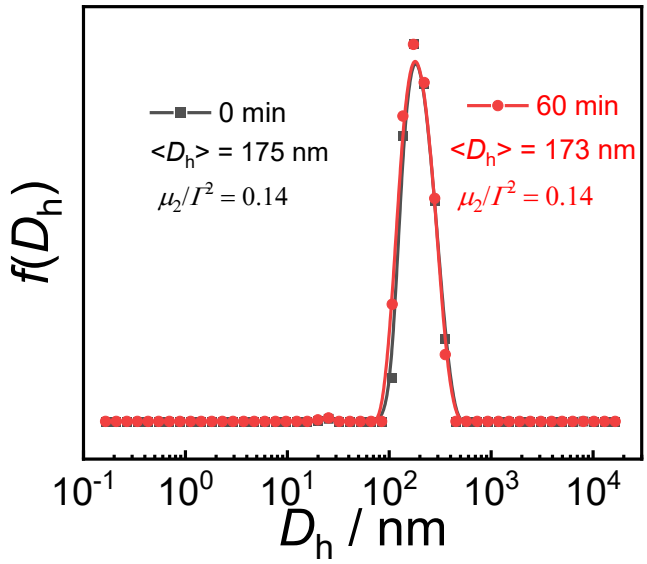

Figure S8. Intensity-average hydrodynamic diameter distributions, $f(D_h)$, of aqueous dispersions (0.1 g/L) of PTNO1 micelles with or without 410 nm irradiation (28 mW/cm^2) for 60 min.

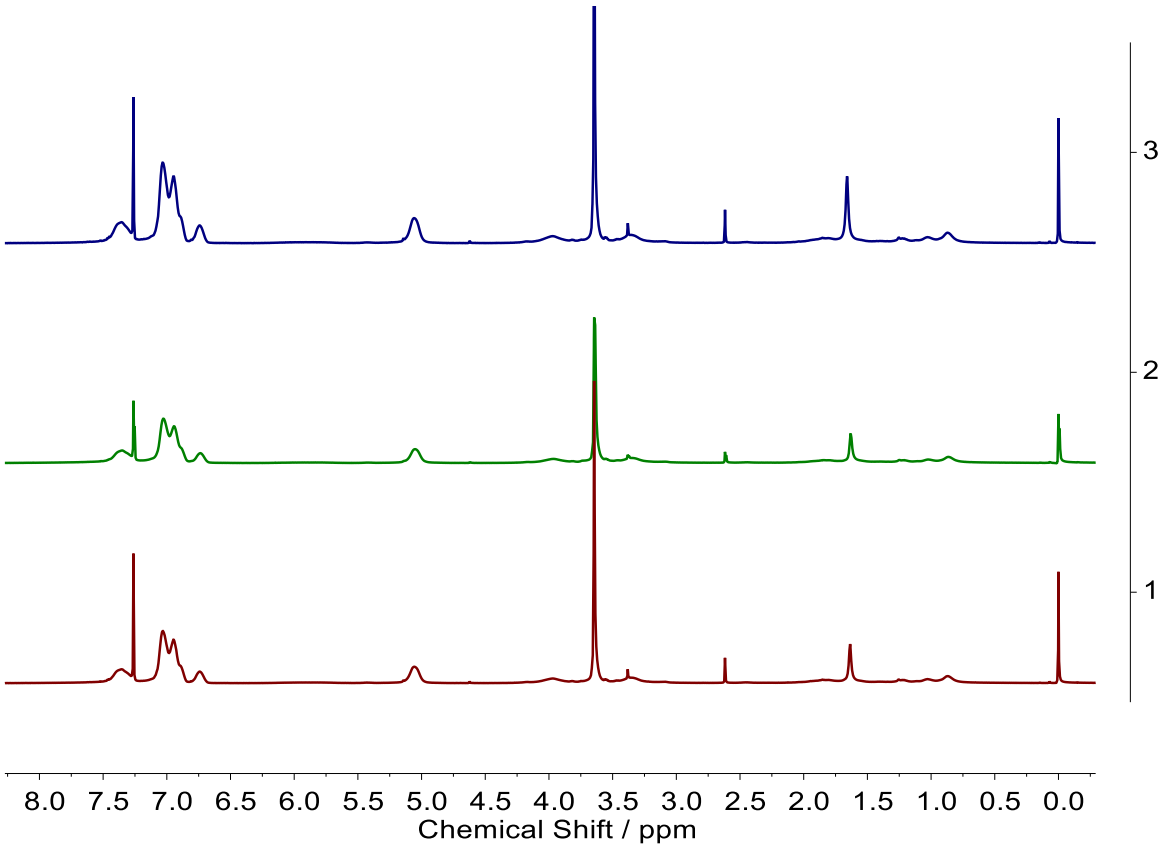

Figure S9. ^{1}H NMR spectra recorded for PTNO1 copolymer upon incubation at room temperature without light irradiation for 0 (top), 24 (middle), and 48 h (bottom), respectively.

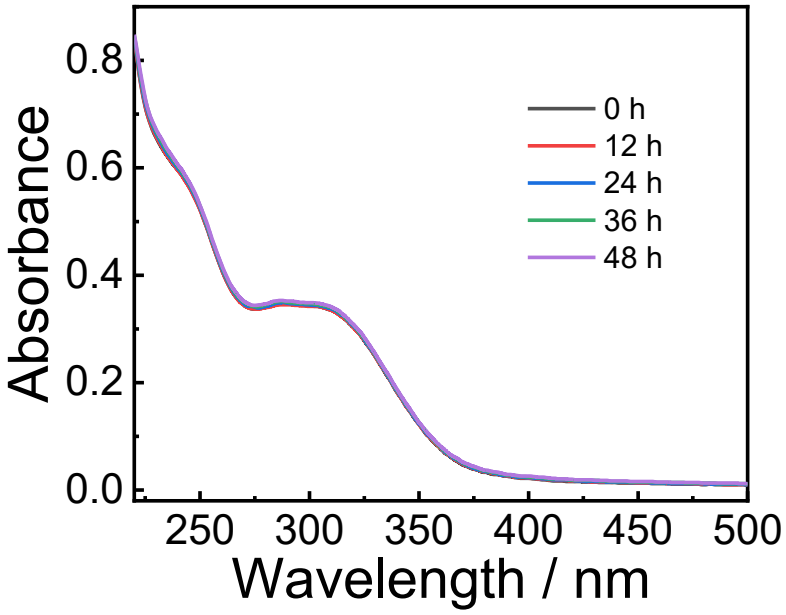

Figure S10. Evolution of UV-vis absorbance spectra of the aqueous dispersion (0.1 g/L) of PTNO1 micellar nanoparticles upon incubation at room temperature without light irradiation.

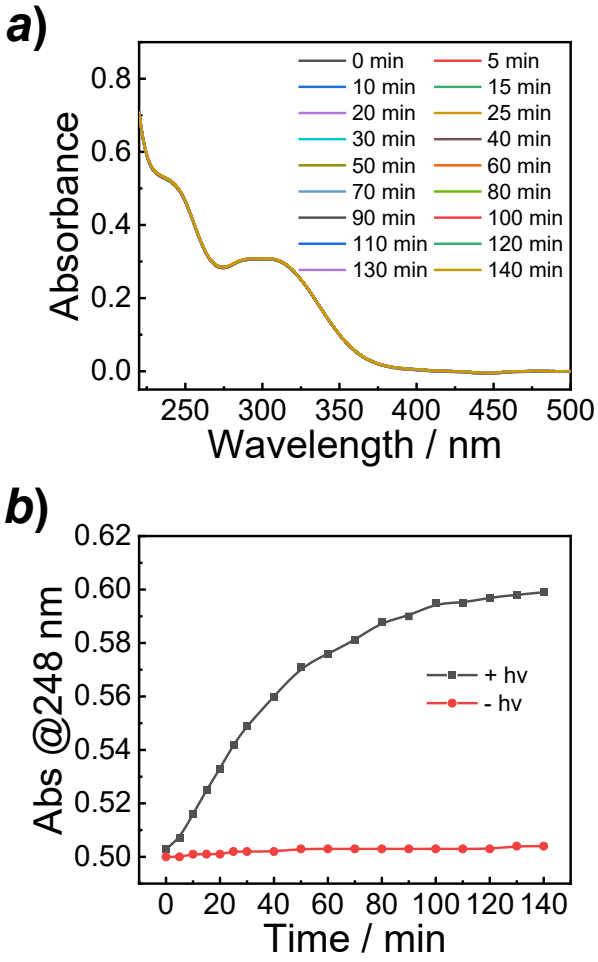

Figure S11. (a) UV-vis spectra of aqueous dispersions (0.1 g/L) of PTNO1 micelles without 410 nm light irradiation. (b) Absorbance intensity changes of aqueous dispersions (0.1 g/L) of PTNO1 micelles with or without 410 nm light irradiation (28 mW/cm^2).

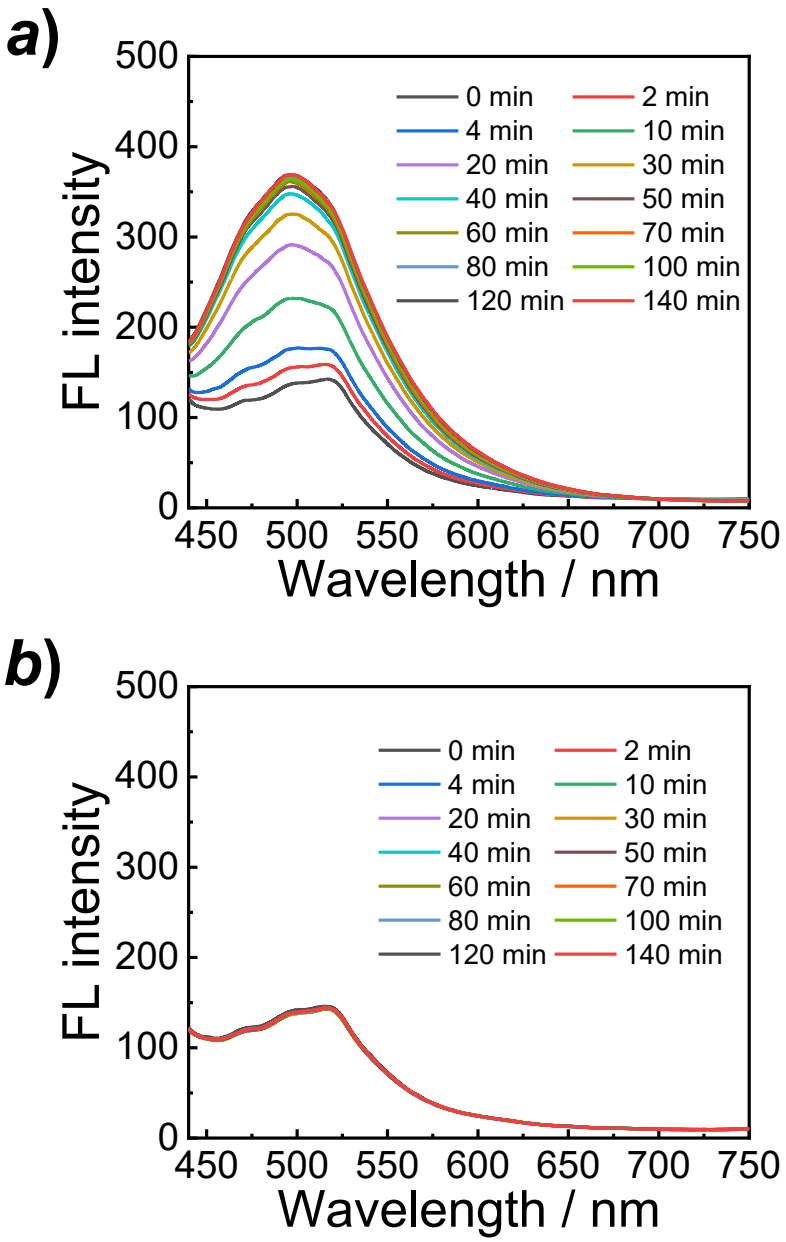

Figure S12. (a,b) Fluorescence emission spectra (λ_{ex} = 405 nm) of aqueous dispersions (0.1 g/L) of PTNO1 micelles (a) with and (b) without 410 nm light irradiation (28 mW/cm^2).

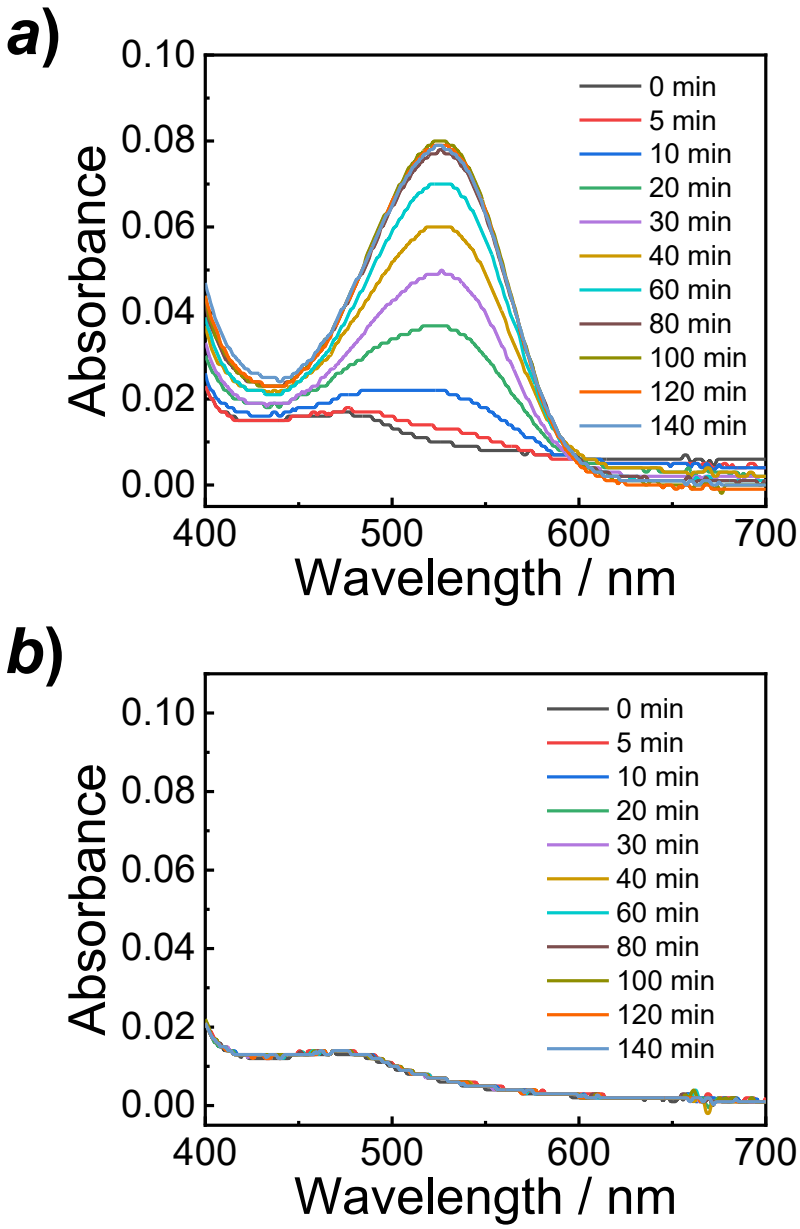

Figure S13. Absorbance spectra of the aqueous dispersions (0.1 g/L) of PTNO1 micelles (a) with or (b) without 410 nm light irradiation (28 mW/cm^2) as assayed by Griess reagent.

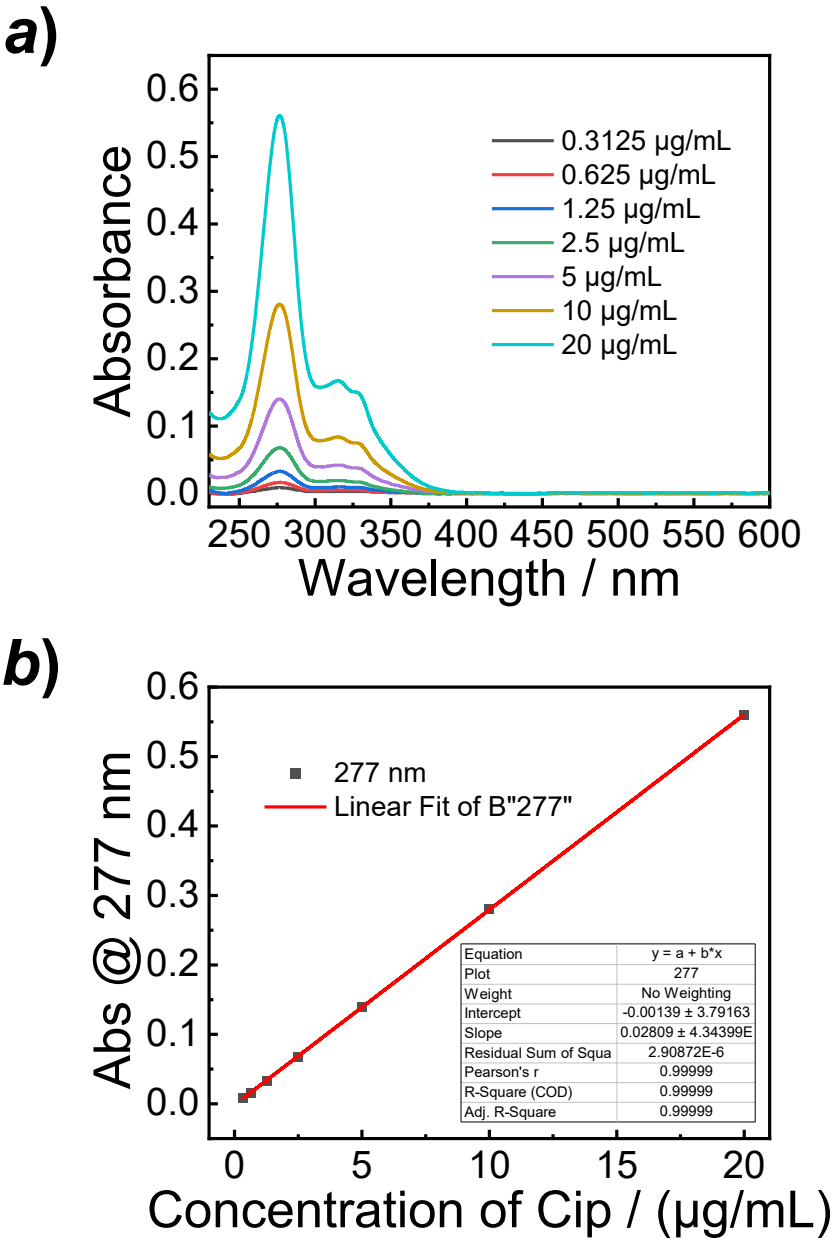

Figure S14. Concentration-dependent (a) UV-vis absorbance spectra and (b) absorbance intensity changes at 277 nm of aqueous solutions (pH 2.0) of ciprofloxacin (Cip).

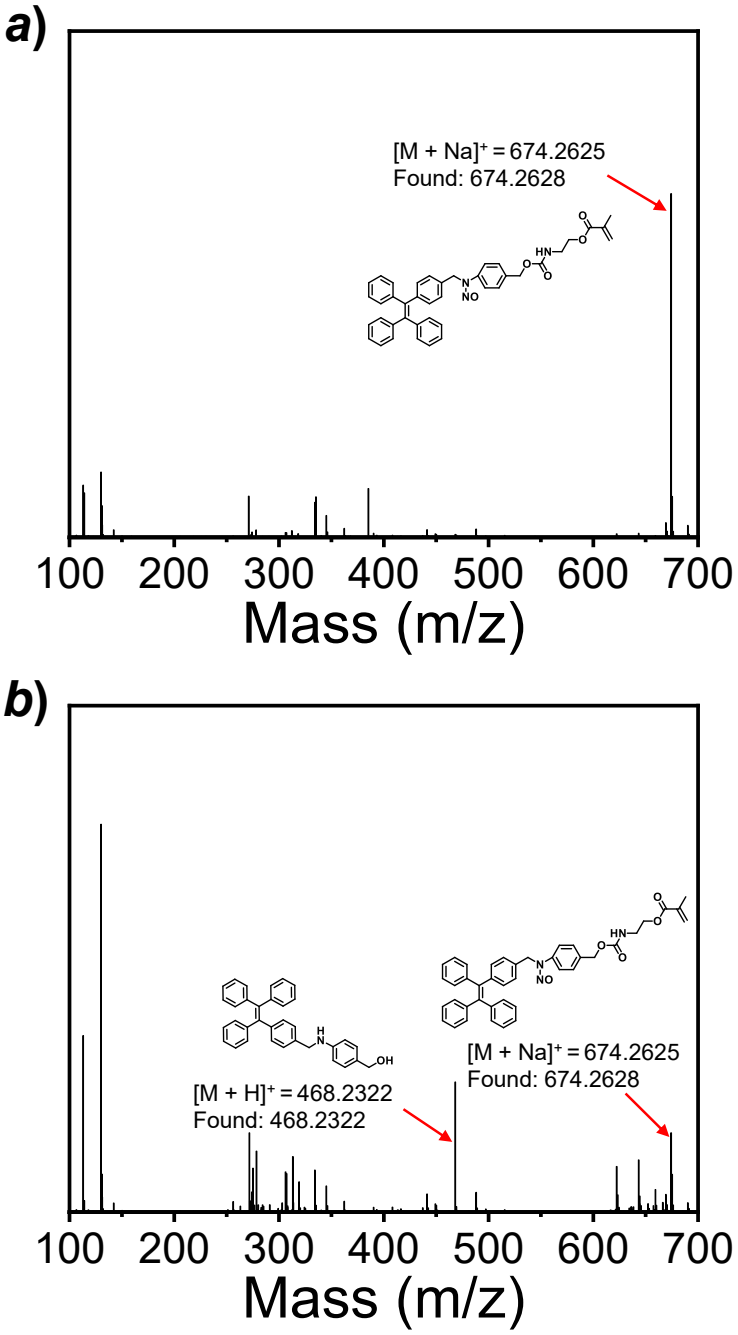

Figure S15. Evolution of ESI-MS of compound ***1*** under 410 nm light irradiation (28 mW/cm^2) for (a) 0 min (b) 30 min, respectively.

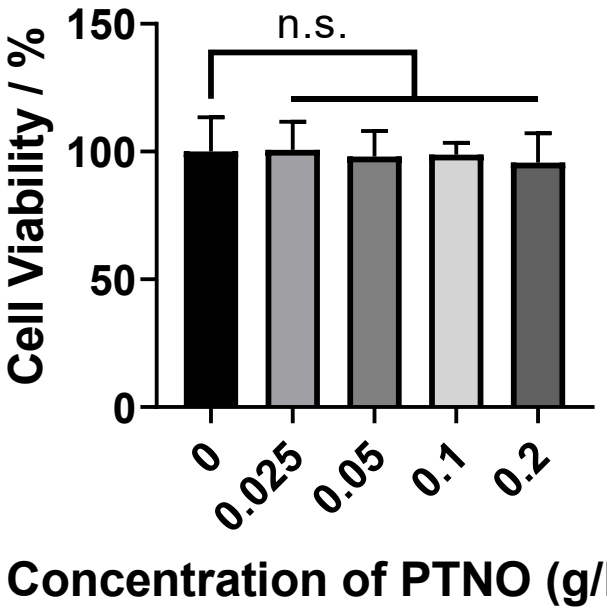

Figure S16. Cell viability of L929 cells as determined by MTT assay after 24 h incubation of PTNO1 micelles at varying concentrations (0-0.2 g/L). Data are shown as mean ± s.d. (n = 5). n.s., not significant, p values were obtained in comparison with the PBS control without the addition of PTNO1 micelles.