Supporting Information

Visible Light-Triggered Self-Reporting Release of Nitric Oxide (NO) for Bacterial Biofilm Dispersal

Zhiqiang Shen,†# Kewu He,‡# Zhanling Ding,† Mengdan Zhang,† Yongqiang Yu,‡* and Jinming Hu†*

† CAS Key Laboratory of Soft Matter Chemistry, Hefei National Laboratory for Physical Science at the Microscale, Department of Polymer Science and Engineering, University of Science and Technology of China, Hefei 230026, Anhui, China;
‡ Department of Radiology, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230022, China

To whom the correspondence should be addressed. E-mail: jmhu@ustc.edu.cn (J.H.); hfyuyongqiang@hotmail.com (Y.Y.).
# These authors contributed equally to this work.
Experimental Section

Materials. 7-amino-4-methylcoumarin (AMC), ciprofloxacin (Cip), and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) were purchased from Energy Chemical. 2-Isocyanatoethyl methacrylate and Griess Reagent were purchased from Sigma-Aldrich. Nile red was purchased from J&K Scientific Co., Ltd. 2,2′-Azobis(2,4-dimethyl-4-methoxyvaleronitrile) (V70) was purified by recrystallization from 98% ethyl acetate and hexane. Luria Bertani medium (Qingdao Hope Bio-Technology Co., Ltd.) and LIVE/DEAD® BacLight™ bacterial viability kit reagents (L-7007, 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-(Hydroxymethyl)benzaldehyde and PEG₄₅-based macroRAFT agent 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.

Sample Preparation. Synthesis of (E)-7-((4-(hydroxymethyl)benzylidene)amino)-4-methyl-2H-chromen-2-one (compound 1, Scheme 2a). 7-Amino-4-methylcoumarin (AMC) (1 g, 5.71 mmol, 1 equiv.) and 4-(hydroxymethyl)benzaldehyde (311 mg, 8.57 mmol, 1.5 equiv.) were dissolved in a mixture of dry ethanol (40 mL) and anhydrous acetonitrile (10 mL). A catalytic amount of glacial acetic acid (100 μL) was added. The reaction mixture was stirred at 90 °C for 16 h. After evaporation of the solvent under vacuum, 30 mL of dry alcohol was added, and stirred at room temperature for 30 min. The residues were filtrated and were washed with ethanol, affording compound 1 as a yellowish solid (1.47 g, yield: 88 %, Figure S1a). The obtained Schiff base was of good purity and was used for next step without further purification procedure.

Synthesis of 7-((4-(Hydroxymethyl)benzyl)amino)-4-methyl-2H-chromen-2-one (Compound 2, Scheme 2a). Compound 1 (1 g, 3.41 mmol, 1 equiv.) was dissolved in a mixture of MeOH and THF (30 mL, v/v = 1/2), and sodium borohydride (NaBH₄, 129.07 mg, 3.412 mmol, 1 equiv.) was slowly added. After the addition was completed, the reaction mixture was stirred for 4 h. Afterward, the residues were further purified by flash chromatography, affording compound 2 as a yellowish solid (0.91 g, yield: 90.2 %; Figure S1b).
Synthesis of N-(4-(Hydroxymethyl)benzyl)-N-(4-methyl-2-oxo-2H-chromen-7-yl)nitrous amide (Compound 3, Scheme 2a). Compound 2 (0.5 g, 1.7 mmol, 1 equiv.) was dissolved in a mixture of THF and glacial acetic acid (20 mL, v/v = 1/1), and aqueous solution (10 mL) of sodium nitrite (NaNO₂, 140 mg, 2.03 mmol, 1.2 equiv.) was slowly added. After the addition was completed, the mixture was further stirred for 2 h. The precipitation was collected by filtration and was washed with DI water, affording compound 3 as a slightly yellow solid (0.505 g, yield: 92%; Figure S1c).

Synthesis of 2-(((4-Methyl-2-oxo-2H-chromen-7-yl)(nitroso)amino)methyl)benzyl oxy)carbonyl)amino)ethyl methacrylate (CouNO, Scheme 2a). Compound 3 (0.5 g, 1.54 mmol, 1 equiv.) was dissolved in THF (20 mL), and a catalytic amount of dibutyltin dilaurate (DBTL, 50 μL) was added. After that, 2-isocyanatoethyl methacrylate (0.287 g, 1.851 mmol, 1.2 equiv.) was added into the mixture under magnetic stirring at room temperature for 10 h. After removing all the solvents, the crude product was further purified by flash chromatography using dichloromethane/methanol (v/v, 99:1) as the eluent to obtain a slightly yellow solid (0.66 g, yield: 89%; Figure S2).

Synthesis of PEO₄₅-b-PCouNO₁₄ Diblock Copolymers (Scheme 2b). Typical procedures employed for the RAFT synthesis of PEO-b-PCouNO diblock copolymers are as follows. CouNO monomer (0.216 g, 0.45 mmol, 15 equiv.), V70 (1.4 mg, 0.0045mmol, 0.15 equiv.), PEO-based macroRAFT agent (67.4 mg, 0.03 mmol, 1 equiv.), and DMSO (0.8 mL) 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 that, the reaction tube was heated at 37 °C for 15 h to proceed the polymerization reaction. The reaction tube was quenched, opened, and diluted with THF. The THF solution was then precipitated into an excess amount of diethyl ether to obtain the PEO-b-PCouNO diblock copolymer. The above dissolution-precipitation cycle was repeated twice. The final product was dried in a vacuum oven, affording PEO-b-PCouNO as a yellowish solid (92 mg, yield: 32.3%). The degree of polymerization, DP, of CouNO block was determined to be 14 according to ¹H NMR analysis in DMSO-d₆ (Figure S3). Thus, the resulting diblock copolymer was denoted as PEO₄₅-b-PCouNO₁₄ (Figure S3a). GPC analysis revealed an $M_n$ of 5.9 kDa and an $M_w/M_n$ of 1.09 (Figure S3b).
Self-Assembly of Diblock Copolymers and the Fabrication of NR/Cip-Loaded Micelles. PEO\textsubscript{45}-b-PCouNO\textsubscript{14} diblock copolymer (1 mg) was dissolved in 1 mL of THF, which was quickly injected into 9 mL of DI water in one shot. After that, THF was removed by dialyzed against DI water. For the NR loading, PEO\textsubscript{45}-b-PCouNO\textsubscript{14} diblock copolymer and Nile red (NR) were dissolved in THF (1 mL), and the final concentrations were 0.1 g/L and 0.01 g/L, respectively. The mixture was then injected into 9 mL of DI water, and the as-assembled NR-loaded PEO\textsubscript{45}-b-PCouNO\textsubscript{14} micelles were purified by dialysis against DI water. For the preparation of Cip-loaded PEO\textsubscript{45}-b-PCouNO\textsubscript{14} micelles, ciprofloxacin (Cip) (0.5 mg) and PEO\textsubscript{45}-b-PCouNO\textsubscript{14} (2 mg) were dissolved in 1 mL of THF, and the THF solution was quickly injected into 4 mL of DI water. The THF was then removed by dialysis, and the resulting Cip-loaded micelles were stored at 4 °C for further use.

Determination of the Critical Micellization Concentration (CMC) of the Diblock Copolymer by Surface Tension Measurement. The variations of the surface tension were monitored in a tensiometer (Krüss Tensiometer K9, Germany) using the Wilhelmy plate method. The aqueous solutions of PEO\textsubscript{45}-b-PCouNO\textsubscript{14} diblock copolymer were held in a thermostated flask with a volume of 20 mL and the temperature was set at 25 °C. The values were averaged through three consecutive measurements.

Visible Light-Mediated NR/Cip Release. NR/Cip-loaded micelles (0.4 mg/mL; 0.8 mL) were placed in sample vials (30 mL) and irradiated with a 410 nm LED lamp (7.5 mW/cm\textsuperscript{2}) for 10 min or 20 min. The same micellar solution without light irradiation was used as the control. The three micelle solutions were dialyzed (MWCO = 14 KDa) against acetic acid buffer (6 mL, pH 5.5) at 37 °C under constant stirring. At predetermined time points, the dialysates were replaced with 6 mL of fresh acetic acid buffer solution. The dialysates were subjected to lyophilization and the residues were dissolved in aqueous solution at pH 2.0. The loading and releasing contents of Cip were calculated against a standard calibration curve of Cip at 277 nm established in a purely aqueous solution at pH 2.0 (Figure S10).

In vitro Cytotoxicity Determination Against HeLa Cells and Normal Human Lung Fibroblast Cells (NHLF). The viability of cell lines was determined by an Alamar blue (AB) assay. HeLa and NHLF cell lines were grown according to the manufacture’s protocol in a 96-well plate. The initial cell concentrations were set at $1 \times 10^5$ cells/mL. The resulting different
cell suspensions were seeded into a 96-well plate (200 μL per well) with PEO_{45-b}-PCouNO_{14} at varying concentrations (0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, and 0.4 g/L) and incubated at 37 °C for 24 h and 48 h, respectively. After removing the media, 110 μL of fresh culture media containing 10 μL of AB solution was added into each well. After incubating at 37 °C for 4 h, the excitation and emission wavelengths were set at 550 nm and 590 nm, respectively, which was recorded on a plate reader (Thermo Fisher Scientific) using 550 nm excitation. The cell viability was calculated using the following equation:

\[
\text{Cell Viability / } % = \frac{(\text{FL}_{\text{sample}} - \text{FL}_{\text{blank}})}{(\text{FL}_{\text{control}} - \text{FL}_{\text{blank}})} \times 100\%
\]

Where FL_{sample} is the fluorescence intensity of the solution in the well where the cells were treated with PEO_{45-b}-PCouNO_{14}; FL_{blank} is the fluorescence intensity of the cell culture media without cells; and FL_{control} is the fluorescence intensity of the solution in the well in which the cells were treated with the medium only.

**Observation of the NO Release by Confocal Laser Scanning Microscopy (CLSM).** The laboratory *P. aeruginosa* PAO1 strain was used. A single colony of PAO1 was inoculated in 4 mL of Luria Bertani (LB) medium at 37 °C with shaking at 200 rpm overnight. 40 μL of the culture medium was withdrawn and was diluted with 4 mL of LB medium, which was further incubated at 37 °C with shaking at 200 rpm for 3 h. 200 μL of the culture medium was added into each well in a 96-well plate, followed by the addition of 100 μL of PEO_{45-b}-PCouNO_{14} micelle dispersion (0.4 g/L). The mixture was further incubated at 37 °C for 12 h with shaking at 200 rpm. Afterward, the culture medium was irradiated with 410 nm LED lamp (7.5 mW/cm²) for 10 min or 20 min; the culture medium without 410 nm light irradiation was used as the control. The irradiated and non-irradiated culture media were subjected to centrifugation at 13000 rpm for 5 min, and the bacteria were collected, re-dispersed with 1 mL of deionized water. The centrifugation and re-dispersion cycle was repeated two times. Finally, the bacteria were dispersed in 0.2 mL of deionized water and were imaged by CLSM. The blue channel was excited at 405 nm and was collected between 410 nm and 460 nm. The green channel was excited at 488 nm and was collected between 500 nm and 560 nm (Figure 5).

**Visible Light-Mediated NO Release for Biofilm Dispersal.** The laboratory *P. aeruginosa* PAO1 strain was used to grow biofilm. In all assays, a single colony of PAO1 was inoculated in 10 mL of Luria Bertani medium at 37 °C with shaking at 200 rpm overnight. The culture
medium was diluted with 1 mL of M9 minimal medium (containing 48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, 2 mM MgSO₄, 20 mM glucose, 100 mM CaCl₂, pH 7.0) to an OD₆₀₀ of 0.005. The diluted bacteria were further incubated at 37 °C in a 24-well plate (Thermo Fisher). The plates were shaking at 80 rpm in an orbital shaker and the biofilms were allowed to grow for 7 h or 24 h without any disruption.

To investigate the antibiofilm effect of PEO₄₅-b-PCouNO₁₄ micellar nanoparticles, preformed PAO1 biofilms were treated with incubated with the micellar nanoparticles for 30 min (37 °C, 120 rpm). After that, the biofilm was irradiated with 410 nm LED lamp for varying times (0 min, 20 min) and the irradiated biofilms were further incubated for 30 min before quantification of the biofilm biomass. Control samples (without micellar nanoparticles and/or without Cip drug loading) were detected as well at the otherwise identical conditions.

**Quantification of the Biofilm Biomass by Crystal Violet Staining.** Biofilm biomass was quantified using the crystal violet (CV) staining method. Briefly, after treatment, the culture supernatant was removed and the biofilm on the well surfaces was washed once with 1 mL of PBS, followed by the addition of 1 mL of the CV staining agent. The plates were incubated on the bench for 20 min before the wells were washed twice with PBS. The CV stained biofilms were mixed with 1 mL 100% ethanol and were quantified by measuring the OD₅₅₀ 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 ATP Assay.** The BacTiter-Glo microbial cell viability assay (Promega) was used for bacterial viability measurements, which is based on the quantitation of the ATP present in bacteria. After the incubation with various treatments, the planktonic dispersions were directly mixed with the BacTiter-Glo reagent following the manufacturer's instructions. After 5 min incubation, the luminescence was measured by using a multimode microtitre plate reader (Thermo Fisher). To evaluate the viability of biofilms, biofilms on the interior surfaces of the wells were first washed twice with PBS before being resuspended and homogenized in PBS by incubating in an ultrasonication bath (200 W, 40 kHz) for 15 min. This resuspension protocol was used similarly for analyzing colony-forming units (CFU) from biofilms. Re-suspended biofilm cells were then mixed with BacTiter-Glo reagent, and their viabilities were quantified by the luminescence measurement as described above.
Evaluation of the Bacterial Viability by Colony Forming Unit (CFU) Assay. The bacterial viability after the treatment were also evaluated by a CFU assay as well. Briefly, PAO1 biofilms with 24 h incubation were grown in the same manner as stated above. Free Cip, PEO₄₅-b-PCouNO₁₄ micellar nanoparticles, and Cip-loaded PEO₄₅-b-PCouNO₁₄ micelles were then added, and the biofilms were incubated at 37 °C with shaking at 200 rpm for 30 min. The treated biofilms were irradiated or not irradiated for 20 min with an LED lamp (410 nm, 7.5 mW/cm²). After that, the biofilms were incubated at 37 °C for 30 min. Afterward, the planktonic and biofilm viability were evaluated by a drop plate method. For planktonic analysis, free-floating cells in the biofilm supernatant were serially diluted in PBS and were plated onto LB agar at 37 °C with shaking at 200 rpm. For biofilm analysis, the residual cells in the interior surface of the well were washed twice with PBS; the cells were homogenized by ultrasound (200 W, 40 kHz) for 15 min, resuspended in PBS, and serially diluted and plated onto LB agar. Planktonic and biofilm colonies were counted, and CFU was calculated after 24 h incubation at 37 °C. All assays included three replicates and were repeated in three independent experiments.

Confocal Laser Scanning Microscopy (CLSM) Observation of the Bacterial Biofilms. For CLSM analysis, *P. aeruginosa* biofilms were grown in glass-bottom, 24-well plates as described above. The formed biofilms were rinsed twice with PBS before being stained with LIVE/DEAD® BacLight™ bacterial viability kit reagents (L-7007, Molecular Probes) according to the manufacturer's instructions. The bacterial films were incubated at room temperature in the dark for 20 min. The samples were then observed with a Leica TCS SP5 microscope. Bacteria that appeared green were considered to be viable, and those stained red and yellow (e.g., overlay of green and red channels) were deemed to be non-viable. The green emission of *P. aeruginosa* bacteria was excited at 488 nm and collected between 500 and 560 nm; the CLSM imaging of SYTO9 was excited at 488 nm and was collected between 490 and 540 nm; the CLSM imaging of PI was excited at 488 nm and collected between 600 nm and 670 nm.

Statistical Analysis. Data are presented as mean ± standard deviations and were analyzed using Prism 8.0 software (GraphPad, San Diego, California, USA) and ordinary two-way ANOVA analysis. The level of significance was set at *p* < 0.05.
Characterization

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV400 NMR (400 MHz) spectrometer operated in the Fourier transform mode. Deuterated dimethyl sulfone (DMSO-$d_6$) was used as the solvent. 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. UV/Vis spectra were recorded on a TU-1910 double-beam UV-vis spectrophotometer (Puxi General Instrumental Company, China). Fluorescence spectra were performed on an F-4600 (Hitachi) spectrofluorometer. Dynamic laser light scattering (LLS) measurements were conducted on a commercial spectrometer (ALV/CGS-3, Langen/Hessen, Germany) and a laser ($\lambda_0 = 632.8$ nm) was used as the light source. The scattered light was collected at a fixed angle of 90° for the duration of ~5 min. All data were averaged over three consecutive measurements. Transmission electron microscopy (TEM) observations were performed on a JEM-2100 electron microscope (JEOL Ltd.) at an acceleration voltage of 200 kV. TEM samples were prepared by placing 10 µL of a mixed solution of assembled aggregates (0.1 g/L) and phosphotungstic acid (2 wt%) on copper grids successively coated with Formvar and carbon films. Confocal laser scanning microscopy (CLSM) images were acquired using a Leica TCS SP5 microscope. Reversed-phase HPLC (RP-HPLC) analysis was performed on a Shimadzu HPLC system, equipped with an LC-20AP binary pump, an SPD-20A UV-Vis detector, and a Symmetry C18 column. Electrospray ionization mass spectrometry (ESI-MS) experiment was performed on Thermo Scientific LTQ Orbitrap Mass Spectrometer equipped with an electrospray interface. Electron paramagnetic resonance (EPR) spectra were recorded a JEOL JES FA200 ESR spectrometer (300 K, 9.063 GHz, Xband) at room temperature. The following parameters were applied, microwave power: 1mW; sweep width ranged from 319.3 to 329.3 mT; modulation frequency: 100 kHz; and modulation amplitude: 0.35 mT.

References


Figure S1. $^1$H NMR spectra recorded in DMSO-$d_6$ for coumarin-based derivatives (a) compound 1, (b) compound 2, and (c) compound 3.
Figure S2. (a) $^1$H and (b) $^{13}$C NMR spectra, (c) ESI-MS, and (d) HPLC elution profile of CouNO monomer.
Figure S3. (a) $^1$H NMR spectrum and (b) GPC elution profiles of PEO$_{45}$-b-PCouNO$_{14}$ diblock copolymer and PEO$_{45}$-based macroRAFT agent.
Figure S4. (a, b) Time-dependent UV-Vis spectra of compound 3 (40 μM) (a) in acetonitrile upon incubation at 60 °C and (b) in a mixture of acetonitrile/H₂O (v/v = 5/95) under ambient light irradiation. (c) Photo-triggered NO release from N-nitrosoamine-based NO donor (compound 3) with the formation of AMC derivative (compound 2). (d) Irradiation duration-dependent HPLC elution profiles detected at 220 nm of 3 under 410 nm light irradiation, confirming the chemical transition from 3 to 2. The asterisk represents the signal of the eluent mixture (MeCN/H₂O = 4/6 (v/v) containing 0.1% TFA)
Figure S5. Evolution of (a) absorbance spectra and (b) absorbance intensity changes of aqueous solution (40 μM) of compound 3 under 410 nm light irradiation. Evolution of (c) fluorescence emission spectra and (d) fluorescence intensity changes ($\lambda_{ex} = 405$ nm, slit width: Ex. 5 nm; Em. 5 nm) of solution (40 μM) of compound 3 under 410 nm light irradiation.
Figure S6. Evolution of (a) absorbance spectra and (b) absorbance intensity changes of aqueous solution (40 μM) of compound 3 without 410 nm light irradiation. Evolution of (c) fluorescence emission spectra and (d) fluorescence intensity changes (λ_ex = 405 nm, slit width: Ex. 5 nm; Em. 5 nm) of solution (40 μM) of compound 3 without 410 nm light irradiation.
Figure S7. Evolution of ESI-MS of CouNO monomer under 410 nm irradiation for (a) 0 min, (b) 20 min, and (c) 50 min, respectively. Photo-mediated NO release was confirmed by the gradual consumption of CouNO monomer, accompanied by the formation CouNH derivative.
Figure S8. (a) Absorbance intensity at 526 nm of Griess reagent as a function of NO$_2^-$ concentration. (b) Absorbance spectra of aqueous dispersion (0.1 g/L) of PEO$_{45}$-b-PCouNO$_{14}$ micellar nanoparticles under 410 nm light irradiation (7.5 mW/cm$^2$) as assayed by Griess reagent.
Figure S9. Irradiation time-dependent (a,b) fluorescence emission spectra and (c) fluorescence intensity changes of Nile red-loaded PEO$_{45}$-b-PCouNO$_{14}$ micellar nanoparticles (a) with or (b) without 410 nm light irradiation (7.5 mW/cm$^2$).
Figure S10. Concentration-dependent (a) absorbance spectra and (b) absorbance intensity changes at 277 nm of Cip in aqueous solution at pH 2.0.
Figure S11. Bacterial biomass analysis by crystal violet staining with various treatments: in the absence of PEO_{45-b-PCouNO_{14}} micelles (36 μg/mL) with 410 nm light irradiation for 20 min; in the presence of PEO_{45-b-PCouNO_{14}} micelles (36 μg/mL) without 410 nm light irradiation and with 410 nm light irradiation for 5 min, 10 min, 15 min, and 20 min, respectively. Data are shown as mean ± S.D. (n = 3; n.s. indicates not significant; *** p < 0.001; **** p < 0.0001). In all cases, the irradiation intensities were 7.5 mW/cm².
Figure S12. (a) Biomass and (b) planktonic biomass of *P. aeruginosa* biofilms treated by aqueous dispersions of PEO₄₅-b-PCouNO₁₄ micellar nanoparticles at varying concentrations under 410 nm light irradiation (7.5 mW/cm²) for 20 min. Data are shown as mean ± S.D. (n = 3; n.s. indicates not significant; *p < 0.05; ***p < 0.001; ****p < 0.0001).
Figure S13. Bacterial activity of (a) planktonic and (b) biofilm treated with free Cip (36 μg/mL), PEO_{45-b}-PCouNO_{14} micelles (36 μg/mL), Cip-loaded PEO_{45-b}-PCouNO_{14} micelles (36 μg/mL) with or without 410 nm light irradiation, respectively, as measured by colony-forming unit (CFU) analysis. Data are shown as mean ± s.d. (n = 4; n.s. indicates not significant; * p < 0.05; *** p < 0.001; **** p < 0.0001).
Figure S14. Plots of HeLa and normal human lung fibroblast (NHLF) cell viability versus PEO45-b-PCouNO14 micelle concentrations (0-0.4 g/L) after incubation for (a) 24 h and (b) 48 h, respectively. Data are shown as mean ± s. d. (n = 5; n.s. indicates not significant).
Figure S15. Time-dependent (a) UV-Vis spectra and (b) absorbance intensity changes at 330 nm of PEG_{45}-b-PCouNO_{14} micellar nanoparticles (0.1 g/L) in DMEM culture medium. (c) Intensity-average hydrodynamic diameters, $<D_h>$, of PEG_{45}-b-PCouNO_{14} micellar nanoparticles (0.1 g/L) in DMEM culture medium. (d) Variations of the surface tension as a function of the PEG_{45}-b-PCouNO_{14} diblock copolymer concentration in aqueous solution.