Non-invasive Diagnosis of Bacterial and Non-Bacterial Inflammations Using a Dual-Enzyme-Responsive Fluorescent Indicator

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# These authors contributed equally to this work.

Contents

1.1 Materials and general methods.

Unless otherwise noted, all reagents and chemicals were obtained from commercial suppliers and used without further purification. 2,3,3-trimethyl-5-nitro-3H-indole, 3-Quinolinecarboxaldehyde, 3,3'-methylenebis (dicoumarol), Methyl trifluoromethanesulphonat (TfOMe) and Pyruvic and Piperazine-1,4-bisethanesulfonic acid (PIPES) were obtained from Bide Pharmatech Ltd. (Shanghai, China). Iodomethane was from Adamas (Shanghai, China). β-Nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH) and β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH) were from Macklin (Shanghai, China). Nitroreductase (NTR) was from Sigma-Aldrich (Shanghai, China). Lipopolysaccharides (LPS) were from Beyotime (Shanghai, China). Test buffer solutions were obtained by mixing 25 mM PIPES and 100 mM NaCl. A Milli-Q system used the purified water in all experiments (Millipore, >18.0 MΩ, USA). The absorbance was recorded by an ultraviolet-visible absorption spectrometer (UV-1900, Shimadzu). Fluorescence spectra were collected on an FS5 fluorescent spectrophotometer (Edinburgh). The NMR spectra were recorded at room temperature with a Bruker Vance III HD 400 MHz spectrometer. The ^{1}H-NMR spectra were recorded at 400 MHz, and the 13C-NMR spectra were recorded at 100 MHz. High-resolution mass spectrum (HRMS) was measured on a 6540 TOF system (Agilent). All pH measurements were performed with a pH-3C digital pH meter (Shanghai ShengCi Device Works, Shanghai, China) with a combined glass-calomel electrode. Fluorescence imaging in cells and zebrafish was performed on a Nikon-Ti2 microscopy imaging system with a 60× or 4× immersion objective lens.

1.2 Cell Culture and MTT assay.

HUVEC cells were plated in 25 cm^2 cell culture flask in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37 ^{o}C in a 5% CO_2 atmosphere. For the MTT assay, HUVEC cells were planted on 96-well flat-bottomed plates (1×10^4 cells per well in 100 μL RPMI-1640), respectively, and allowed to grow for 12 h. Then, they were incubated with fresh culture media (100 μL) containing CyQ at varying concentrations (0, 1.25, 2.5, 5, 10, and 15 μM) at 37 ^{o}C for 24 h. Culture media was removed, and fresh media (100 μL) containing MTT solution (10 μL, 5 mg/mL), followed by incubation at 37 ^{o}C for 4 h. 100 μL of Formazan solvent was added to each well to dissolve the formed formazan. After shaking the plates for 4 h, absorbance values of the wells were read with a microplate reader at 570 nm. The following formula was used to calculate the viability of cell growth: Cells viability (%) = (OD(sample) – OD(blank))/ (OD(control) – OD(blank)) × 100%. Each concentration was tested with 5 wells.

1.3 Preparation of bacteria.

The bacterial strains *Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Acinetobacter baumannii (A. baumannii), Klebsiella pneumoniae (K. pneumonia), Enterococcus faecium (E. faecium) and Pseudomonas aeruginosa (P. aeruginosa)* were used in this study. The six bacterial strains were grown in a Luria-Bertani (LB) medium at a shaker speed of 180 rpm overnight at 37 ^{o}C until the OD_{600} reached 1.0. Then, the cell pellets were centrifuged at 8000 rpm for 1 min and washed thrice with PIPES buffer (pH 7.4). For the toxicity testing of CyQ, the six bacterial strains were cultured in LB (Luria Bertani) liquid medium at 37 ^{o}C until the OD_{600} reached 0.5. Then, the bacteria were diluted with fresh LB liquid medium to an OD_{600} of 0.2. Subsequently, different concentrations of CyQ (0, 2.5, 5, 10, 12, 15 μM) were added to the bacterial solution, and the bacteria were shaken and incubated at 37 ^{o}C. Finally, the OD_{600} of the bacteria was measured and recorded.

1.4 Detection of NAD(P)H and NTR levels in bacteria.

The six bacterial strains were cultured in an LB liquid medium at 37 ^{o}C until the OD_{600} reached 1.0. *S. aureus* was resuspended in the PIPES buffer with an OD_{600} of 0.5. 1 mL aliquots of the bacterial suspensions in microcentrifuge tubes were treated with CyQ (5 μM) at 37 ^{o}C for 0.5, 1, 2, 4 and 12 h to determine the optimal incubated time. Next, *S. aureus* with an OD_{600} of 0.2, 0.5, 1.0, respectively and the other five bacterial strains with an OD_{600} of 0.5 were resuspended in PIPES buffer. The six washed cells were divided into two groups: a control group (control, without treatment) and an inhibitor treatment group (dicoumarol). For the dicoumarol treatment group, cells were pretreated with dicoumarol at a 0.1 mM dose for 1 h. CyQ (5 μM) was added to the mixture and incubated for 4 h. Fluorescence intensity of the bacterial solutions was measured (λ_{ex} = 540 nm).

1.5 Colocalization studies.

Fluorescence imaging was performed using a Nikon inverted research microscope and Nikon A1R HD25 confocal microscope, equipped with CFI Plan Apo × 60 oil (NA = 1.4) objective, X-Light spinning disk module (CREST Optics), and a Hamamatsu Orca Flash 4 ms CMOS camera with a bandpass filter of 379/34 nm (Semrock), 456/48 nm (Semrock), 560/40 nm (Semrock) and 630/60 nm (Semrock). All the images were recorded using NIS Elements and then processed using Fiji software. Pearson's correlation coefficients for colocalization were calculated using a Coloc2 Fiji plugin.

The colocalization analysis of CyQ was conducted in HUVEC cells using Lyso-Tracker green and Mito-Tracker green. Initially, HUVEC cells were cultured in confocal Petri dishes with bovine albumin culture media. The medium was subsequently aspirated, and the cells were washed with 2 mL of PBS per well, repeated three times. Subsequently, HUVEC cells were incubated with 5 μM CyQ and 200 nM Mito-Tracker Green for 15 min at a temperature of 37 °C. Colocalization between Lysosome-Tracker Green (50 nM, 30 min) and DAPI (5 μg/ml, 15 min) was performed under the same condition as Mito-Tracker Green. Fluorescent images were acquired using bandpass filters of 570-640 nm upon excitation at 533-557 nm (CyQ), 512-588 nm upon excitation at 465-495 nm (Mito-Tracker Green and lysosome-

Tracker Green), and 432-480 nm upon excitation at 362-396 nm (DAPI). ImageJ (National Institute of Health) standardized all images to the same brightness level.

1.6 In vitro imaging of NAD(P)H and NTR.

The six bacterial suspensions were cultured in an LB liquid medium at 37 °C until the OD_{600} reached 0.5. Subsequently, the bacteria were washed. Next, the six washed bacterial samples were either pretreated with dicoumarol (0.1 mM) for 1 hour or left untreated, following which they were treated with CyQ (5 μM) for an additional 4 hours. Prior to imaging, the bacterial suspensions underwent washing with PIPES buffer. Imaging of the six bacterial samples was then conducted using a bandpass of 570-640 nm upon excitation at 533-557 nm.

To simulate the inflammatory effect in vitro, HUVEC cells were seeded at a density of 1×10^5 cells per well in a glass Petri dish and incubated with RPMI-1640 without antibiotics or FBS for 24 hours. Concurrently, bacterial strains were cultured overnight in LB to attain the stationary phase. The bacteria were subsequently washed, resuspended in RPMI-1640, and used to infect the cells at various multiplicities of infection (MOI) such as 10, 100, and 1000 for 3 hours. Following this, CyQ (5 μM) was introduced into the wells and incubated for 0.5, 1, and 2 hours, respectively.

Additionally, other HUVEC cells were pre-stimulated with varying concentrations of LPS (0, 0.1, 1, 10 μg/mL) for 24 hours and then treated with CyQ (5 μM) for 2 hours. Subsequently, the cells underwent three washes with PIPES (pH 7.4) to eliminate any remaining probe.

At last, HUVEC cells were incubated with DAPI (5 μg/ml, 15 min) at 37 °C. The cells underwent three washes with PIPES (pH 7.4) to eliminate any residue.

Fluorescence images of HUVEC cells were captured using a Nikon microscope, employing the blue channel with a bandpass of 432-480 nm upon excitation at 362-396 nm and the red channel with a bandpass of 570-640 nm upon excitation at 533-557 nm.

1.7 In vivo imaging of NAD(P)H and NTR.

Initial experiments were conducted on healthy embryos (5 days post-fertilization, dpf) to induce inflammation. Groups of 12 larvae were distributed into 6-well plates, each containing 2.0 mL of E3 medium with or without LPS (25, 50 μg/mL) or an *S. aureus* suspension (approximately 10^7 CFU/mL) as a control. For the LPS treatment groups, embryos were exposed to varying concentrations of LPS (25, 50 μg/mL) dissolved in E3 medium for 36 hours at 28.5 °C. Zebrafish embryos were transferred into a fresh E3 medium containing LPS every 12 hours. For the Infection (*S. aureus* only) treatment groups, bath immersion infections were initiated at 12 hours on zebrafish embryos at 28.5 °C. Subsequently, the embryos developed for up to 24 hours in a fresh E3 medium. Afterward, the groups were incubated with CyQ (5 μM) at 28.5 °C for 2 hours. Following this, the zebrafish were washed with PBS, fixed with paraformaldehyde, and imaged using a Nikon inverted research microscope. Fluorescence intensity was analyzed using ImageJ software, with the red channel set at λ_{ex} = 533-557 nm and λ_{ex} = 570-640 nm.

1.8 Synthetic procedure and characterization of CyQ.

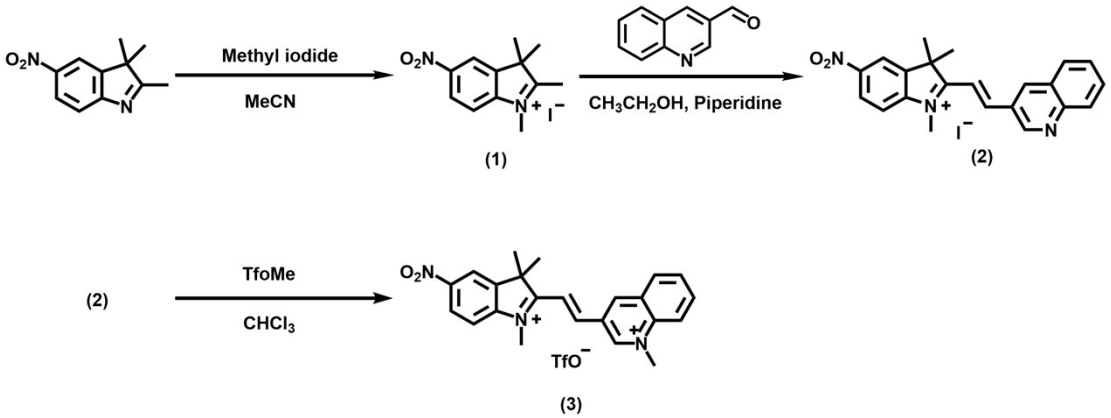

Figure S1. The synthesis procedure for CyQ.

Compound 1. A mixture of 2,3,3-trimethyl-5-nitro-3H-indole (1.00 g, 4.9 mmol) and Iodomethane (1.38 g, 9.8 mmol) was dissolved in acetonitrile (30 mL) and refluxed at 80 °C for 36 hours. The mixture was then cooled to room temperature and concentrated in vacuum. The crude product was filtered upon the addition of 50 mL ether. The resulting precipitate was collected via suction filtration followed by extensive washing with ether to obtain an off-white solid (0.75 g, 44.2% yield). Compound 1 was utilized in the subsequent reaction without undergoing additional purification.

Compound 2. To a mixture of compound 1 (0.50 g, 1.4 mmol) and 3-quinoline carboxaldehyde (0.26 g, 1.73 mmol) in 20 mL anhydrous ethanol was added piperidine (20 μL). Then, the mixture was refluxed for 18 h under a nitrogen atmosphere. After cooling down to room temperature, the precipitate was filtered, washed with cold ether, and dried in vacuum to obtain compound 2 as a red solid (0.62 g, 88.4% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.74 – 9.70 (d, J = 2.1 Hz, 1H), 9.36 – 9.32 (d, J = 2.3 Hz, 1H), 8.95 – 8.90 (d, J = 2.3 Hz, 1H), 8.85 – 8.76 (d, J = 16.7 Hz, 1H), 8.60 – 8.53 (dd, J = 8.8, 2.3 Hz, 1H), 8.23 – 8.19 (d, J = 8.9 Hz, 1H), 8.17 – 8.13 (m, 2H), 8.09 – 8.03 (d, J = 16.5 Hz, 1H), 8.00 – 7.95 (m, 1H), 7.82 – 7.76 (t, J = 7.6 Hz, 1H), 4.31 – 4.27 (s, 3H) ,1.98 – 1.94 (s, 6H).
^{13}C NMR (101 MHz, DMSO-d_6) δ 186.21, 153.08, 151.41, 149.43, 148.45, 146.90, 145.50, 140.20, 133.41, 130.24, 129.60, 128.78, 128.19, 127.66, 125.88, 119.50, 117.18, 115.50, 53.60, 36.10, 25.47.

Compound 3. Methyl trifluoromethanesulphonate (TfOMe, 0.8 g, 4.9 mmol) was added to a suspension of compound 2 (0.5 g, 1.03 mmol) in dry DCM (15 mL) at room temperature under the nitrogen atmosphere. The mixture was stirred for 8 h. Then the precipitate was filtered off, washed with DCM and ether, and the residue was purified by column chromatography (silica gel, CH_2Cl_2: CH_3OH = 5:1, v/v) to obtain pure CyQ as a brownish red solid (0.65 g, 75.4 % yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.90 – 9.86 (s, 1H), 9.42 – 9.38 (d, J = 1.8 Hz, 1H), 8.50 – 8.46 (d, J = 8.9 Hz, 1H), 8.41 – 8.35 (d, J = 8.2 Hz, 1H), 8.27 – 8.21 (ddd, J = 8.8, 7.0, 1.4 Hz, 1H), 8.14 – 8.09 (dd, J = 8.8, 2.4 Hz, 1H), 8.07 – 8.01 (t, J = 7.6 Hz, 1H), 7.97 – 7.89 (d, J = 2.3 Hz, 1H), 7.18 – 7.11 (d, J = 16.1 Hz, 1H), 6.96 – 6.88 (m, 1H), 6.72 – 6.66 (d, J = 8.8 Hz, 1H), 4.67 – 4.62 (s, 3H), 2.92 – 2.86 (d, J = 5.6 Hz, 3H), 1.38 – 1.33 (s, 4H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 154.66, 149.80, 143.06, 138.73, 138.52, 137.70, 135.42, 135.25, 130.73, 130.70, 130.66, 129.55, 126.99, 126.76, 122.71, 119.58, 119.51, 118.47, 116.31, 105.85, 99.68, 48.67, 45.89, 40.53, 40.33, 40.12, 39.91, 39.70, 39.49.

1.9 Preparation of the test solutions.

The UV/Vis absorption and fluorescence responses of CyQ to NADH and NTR were measured in PIPES buffer solutions (25 mM PIPES, 100 mM NaCl, pH 7.4). The stock solution of NTR (1 mg/mL) was prepared Milli-Q. To keep the enzyme activity, all these solutions were stored at -80 °C. NADH stock solution (10 mM) was prepared in PB buffer solutions (50 mM, pH 7.4).

1.10 Determination of the detection limit.

The limit of detection (LOD) was determined by fluorescence titration of the CyQ (5 μM) in the presence of varying concentrations of NADH (0-80 μM) and NTR (0-10 μg/mL). The fluorescence emission spectrum of CyQ was measured and the standard deviation of the blank measurement was obtained. The detection limit was calculated using the following equation:

$$LOD = 3\frac{\sigma}{k}$$

Where σ is the standard deviation of the blank measurement, *k* is the slope between the fluorescence intensity (F 580 nm) versus NADH concentrations and NTR concentrations.

1.11 Statistical Analysis.

The experiments were performed at least three times, and all data were presented as mean ± standard deviation (SD). All figures in this article were generated from three independent experiments that yielded similar results unless otherwise specified. The statistical analysis of the differences between the two groups was conducted using a two-tailed Student's t-test. A significance level of $P \leq 0.05$ (*), $P \leq 0.01$ (**) or $P \leq 0.001$ (***) was considered statistically significant.

2.1 HRMS spectra of compound 1 in DMSO-d_6.

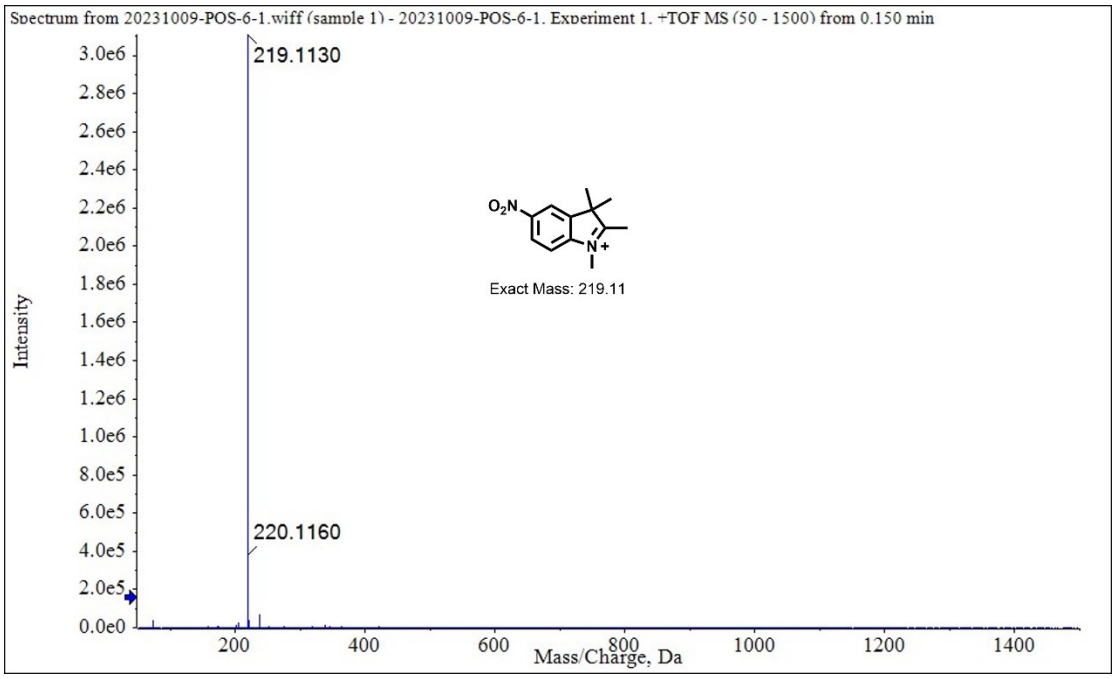

Figure S2. HRMS spectrum of compound 1.

2.2 ^{1}H NMR, ^{13}C NMR and HRMS spectra of compound 2 in DMSO-d_6.

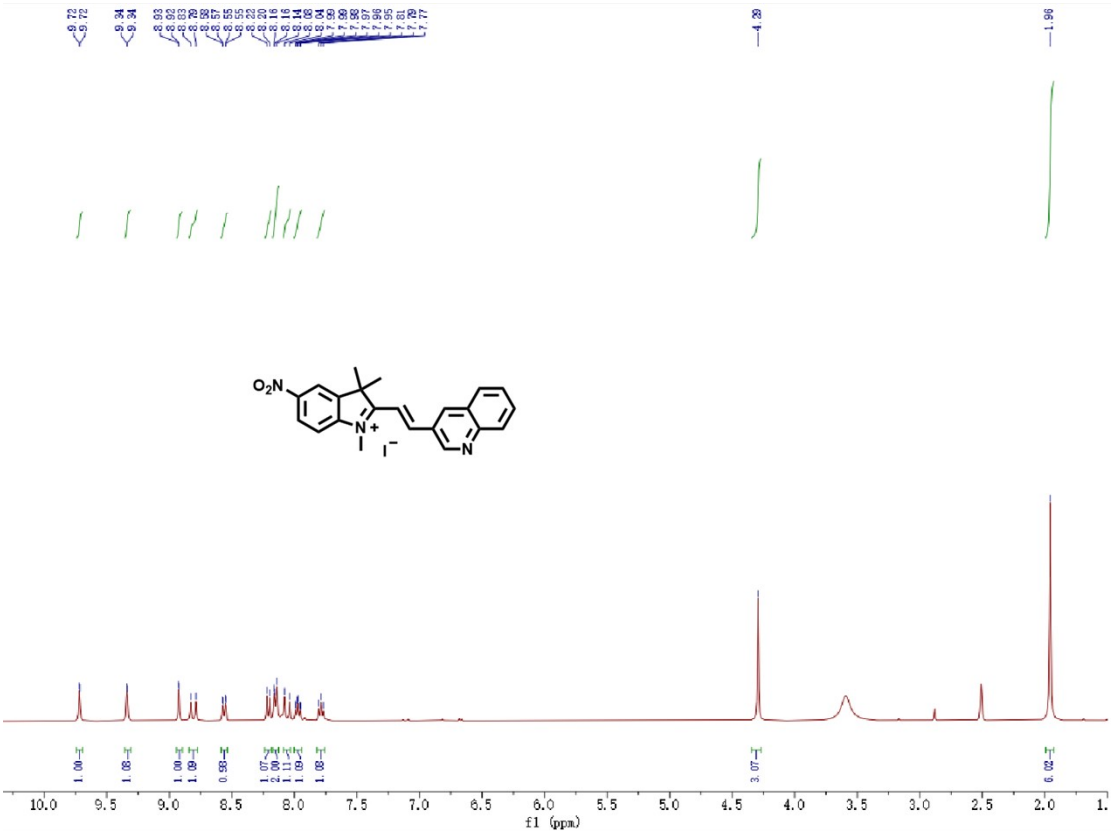

Figure S3. ^{1}H NMR spectrum (400 MHz, **DMSO-d_6**) of compound 2.

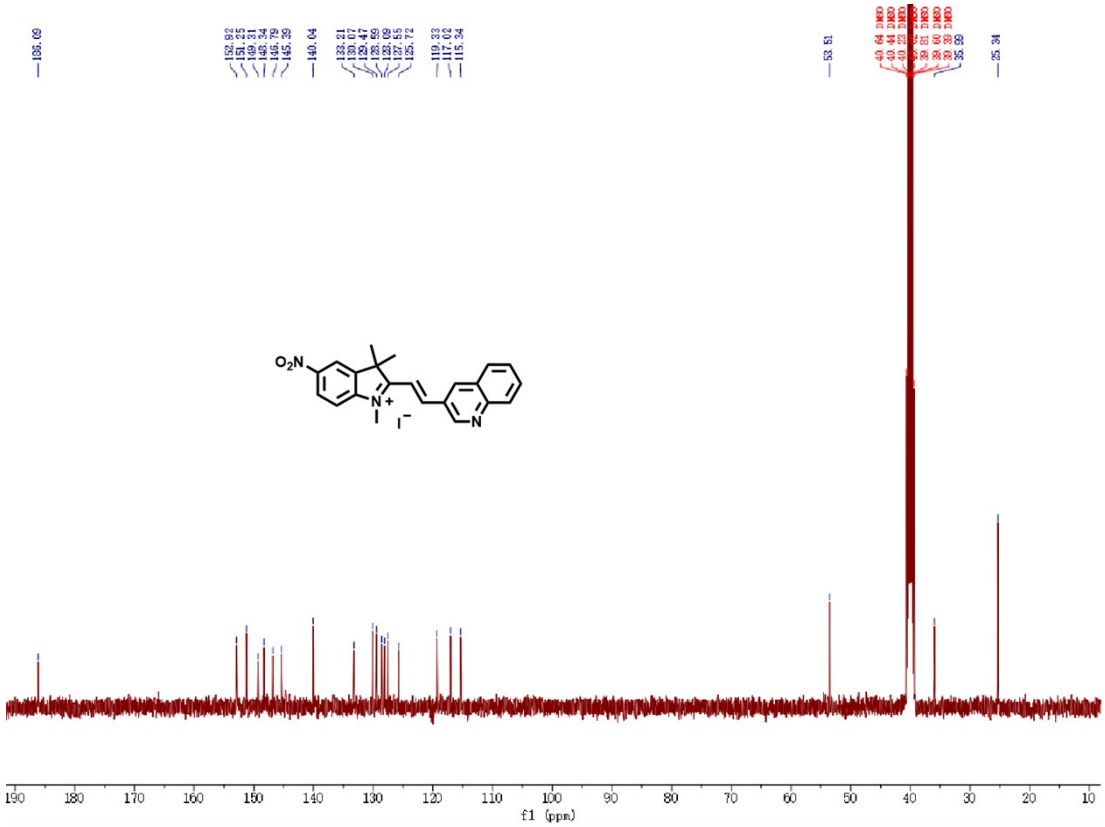

Figure S4. ^{13}C NMR spectrum (100 MHz, **DMSO-d_6**) of compound 2.

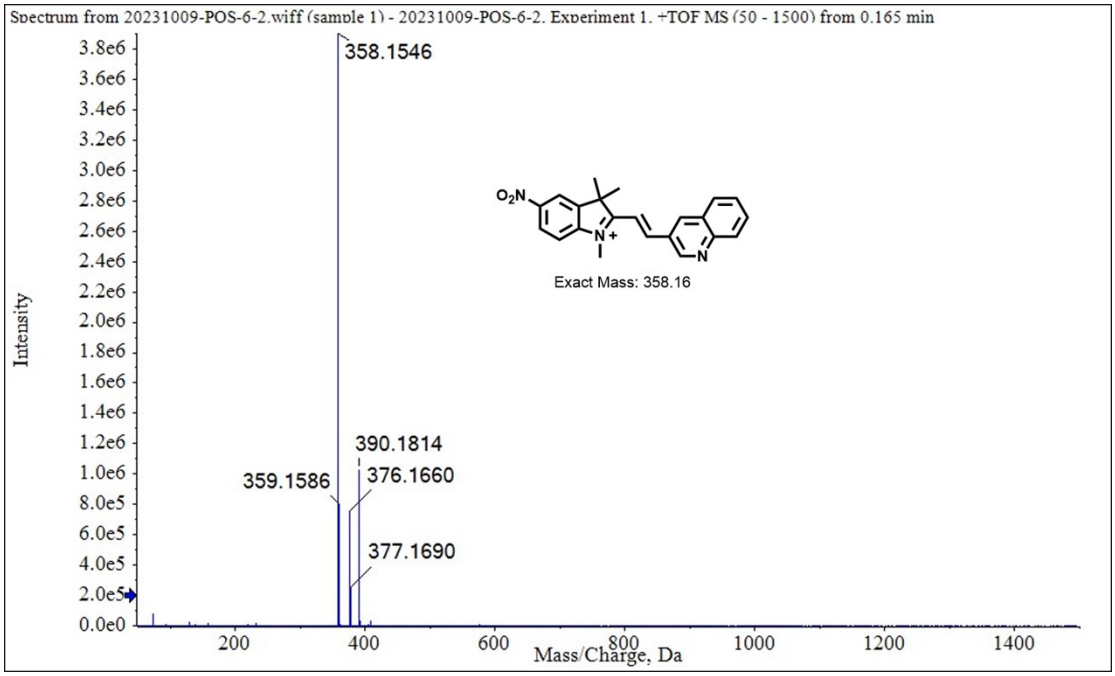

Figure S5. HRMS spectrum of compound 2.

2.3 ^{1}H NMR, ^{13}C NMR and HRMS spectra of CyQ in DMSO-d_6.

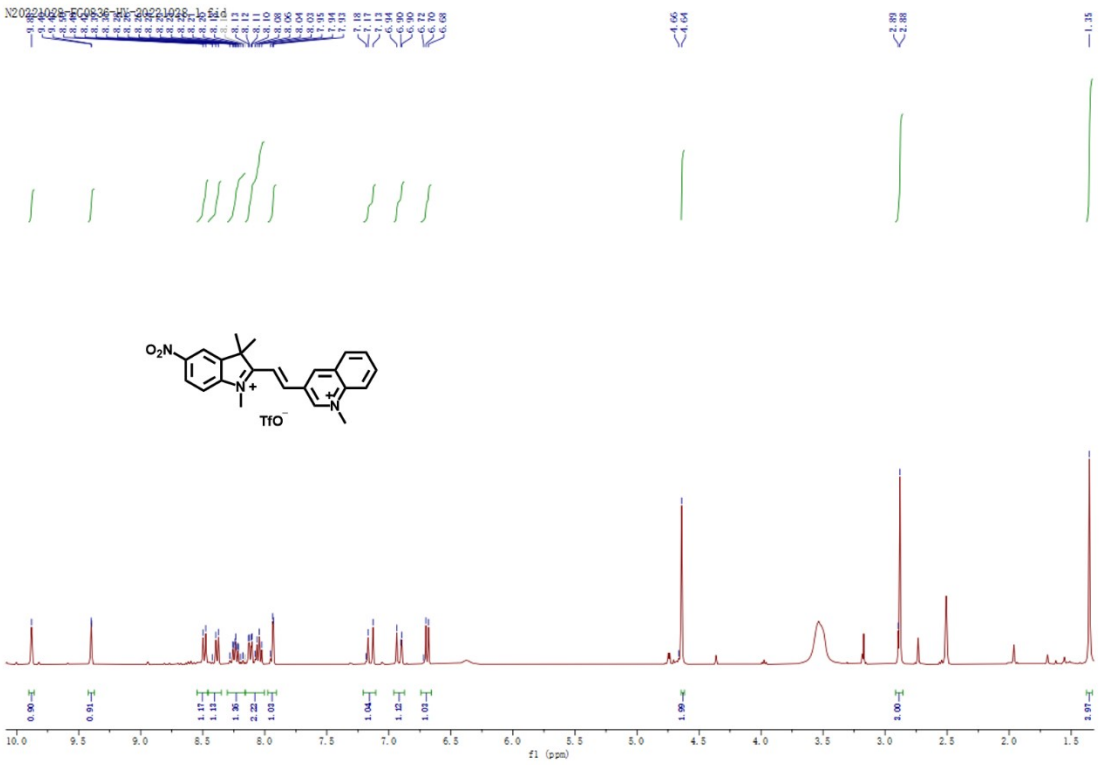

Figure S6. ^{1}H NMR spectrum (400 MHz, **DMSO-d_6**) of CyQ.

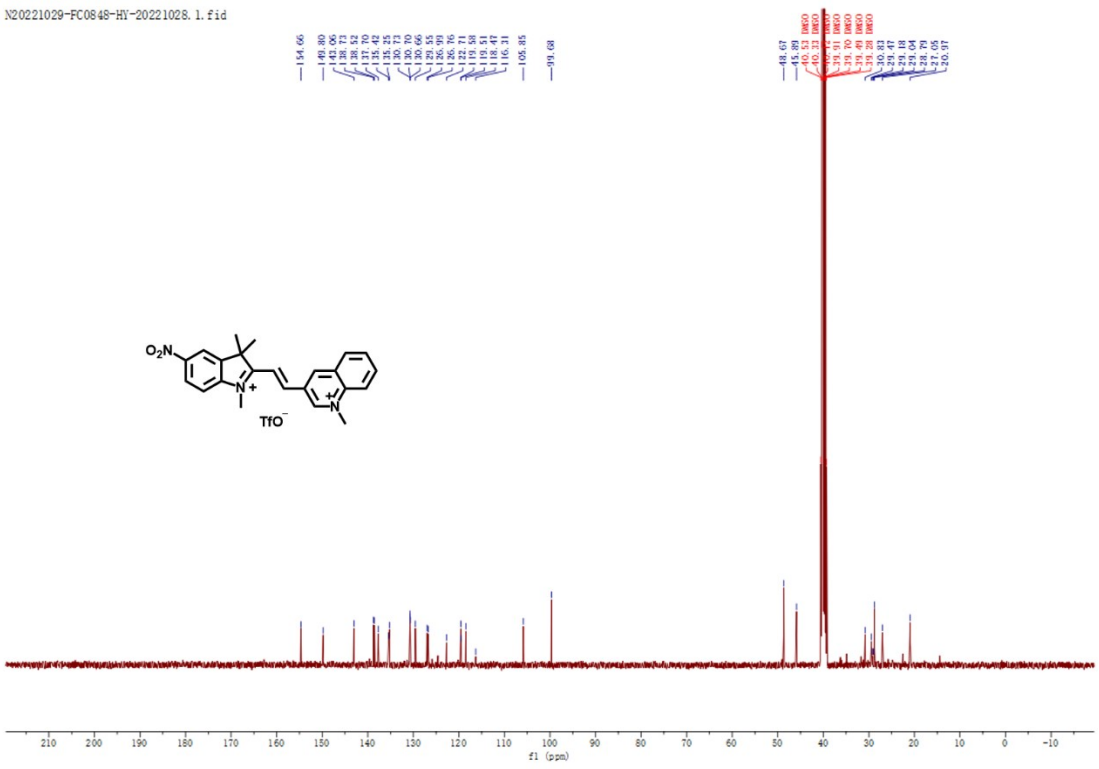

Figure S7. ^{13}C NMR spectrum (100 MHz, **DMSO-d_6**) of CyQ.

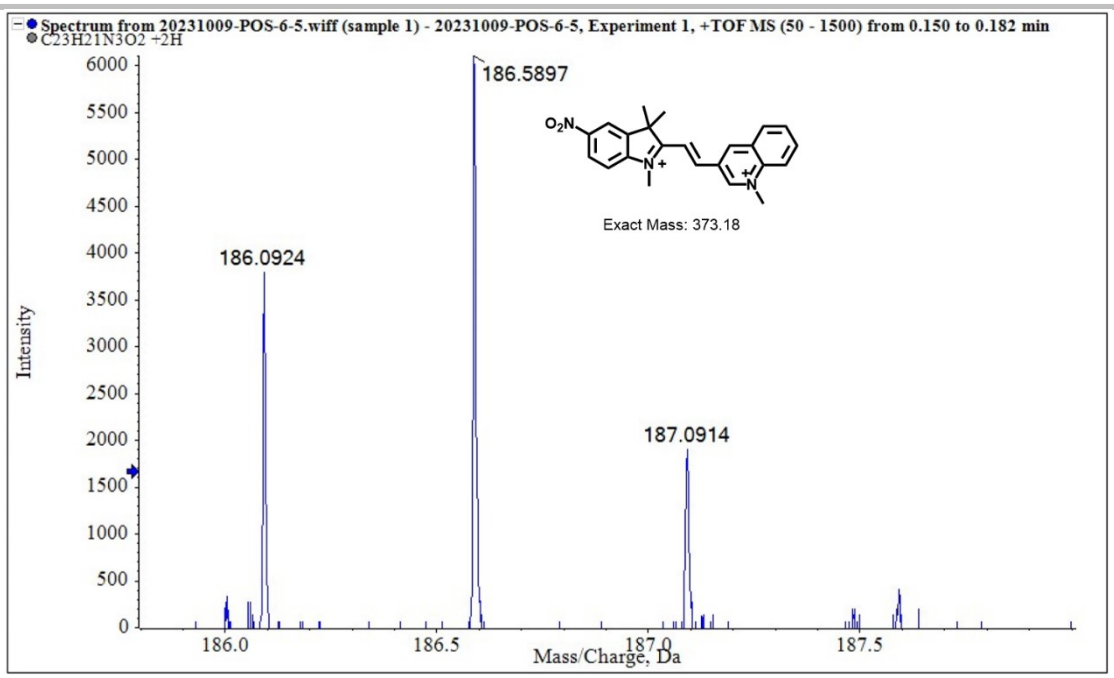

Figure S8. HRMS spectrum of CyQ.

2.4 HRMS for CyQH.

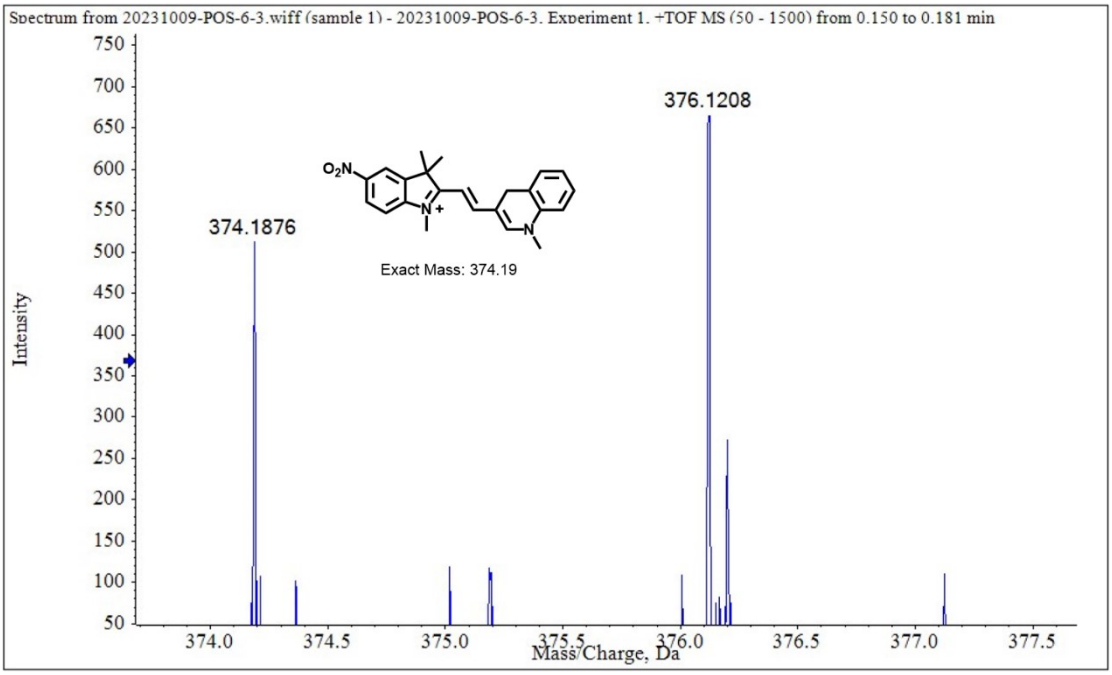

Figure S9. HRMS spectra for CyQH.

2.5 HRMS for CyQN.

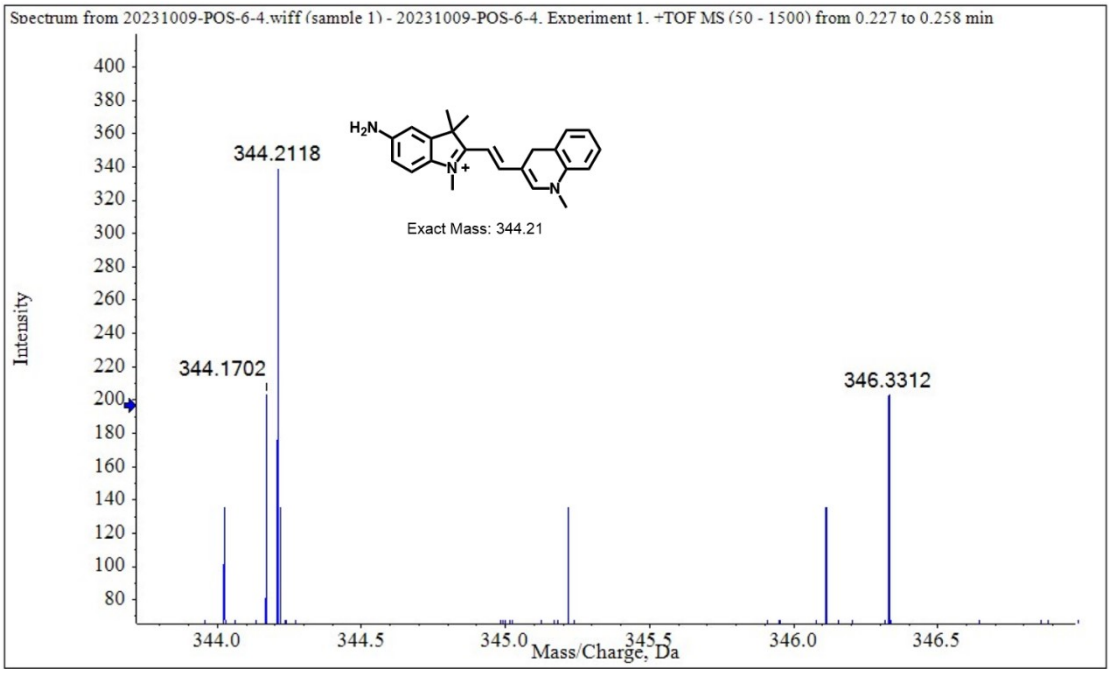

Figure S10. HRMS spectra for CyQN.

2.6 The concentration and time-dependent fluorescence spectra of CyQ upon the addition of NADH.

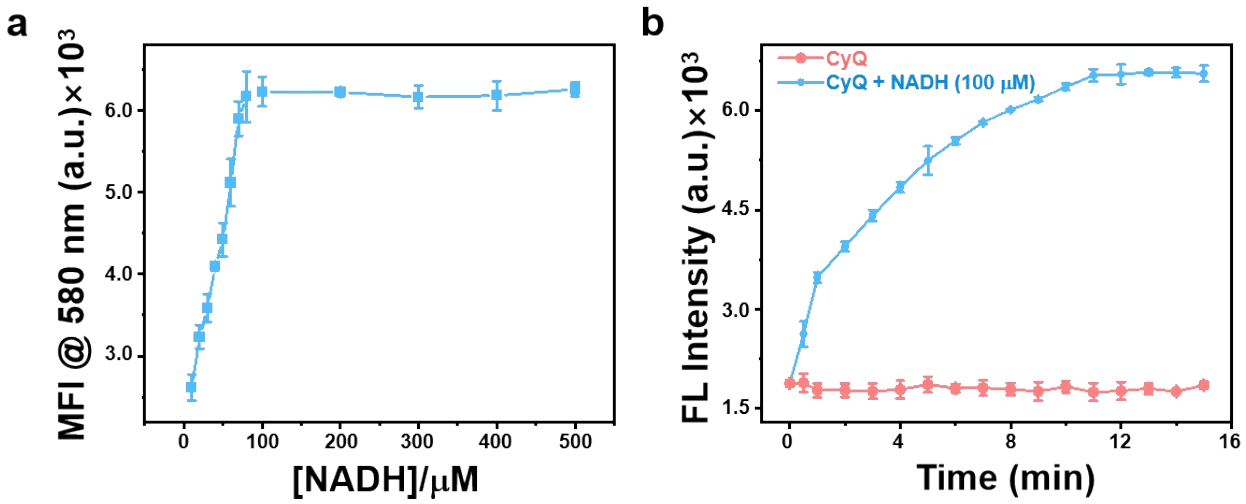

Figure S11. (a) Fluorescence intensity changes of CyQ (5 μM) after the addition of different concentrations of NADH (10-500 μM) in PIPES buffer for 30 min at 37 °C. (b) Time-dependent fluorescence intensity of CyQ (5 μM) at 580 nm in the absence (red) and presence (blue) of NADH (100 μM). All data were recorded in PIPES buffer (25 mM, pH 7.4) at 37 °C, λ_{ex} = 540 nm, λ_{em} = 580 nm (blue) / 574 nm (red). Error bars represent standard deviation (SD, n=3).

2.7 Photostability test.

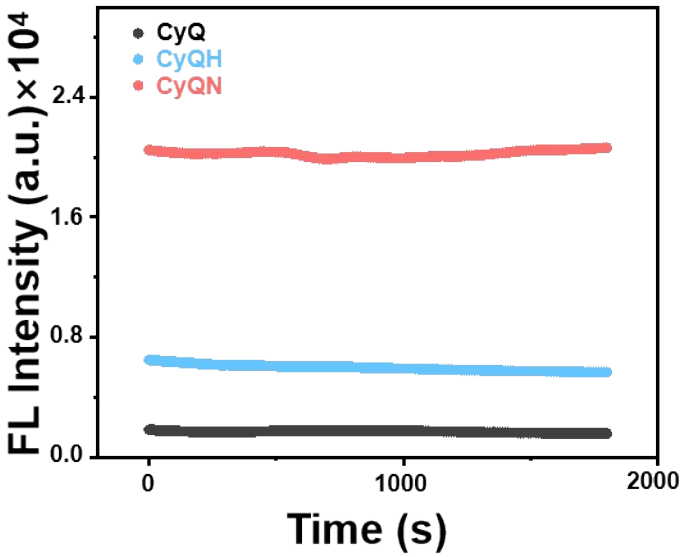

Figure S12. Photostability evaluation of CyQ, CyQH (CyQ + 100 μM NADH) and CyQN (CyQ + 10 μg/mL NTR + 500 μM NADH) under UV irradiation. CyQ, CyQH (CyQ + 100 μM NADH) and CyQN (CyQ + 10 μg/mL NTR + 500 μM NADH) was dissolved in PIPES solution (pH = 7.4) . Fluorescence measurements were recorded at room temperature for 30 min.

2.8 Analysis of fluorescence intensities in bacteria solutions.

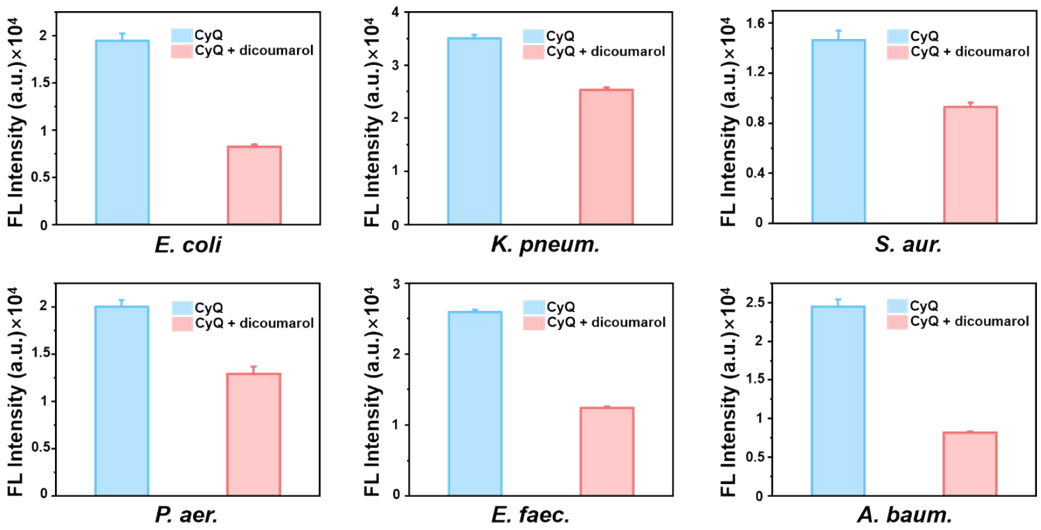

Figure S13. The mean fluorescence intensities analysis for dicoumarol (0 or 0.1 mM, 1h) pretreated *E. coli, S. aureus, E. faecium, K. pneumoniae, P. aeruginosa* and *A. baumannii* bacteria solutions (OD_{600} = 0.5) incubated with CyQ (5 μM, 4h).

2.9 Analysis of fluorescence intensities of *S.aureus* in live and dead states.

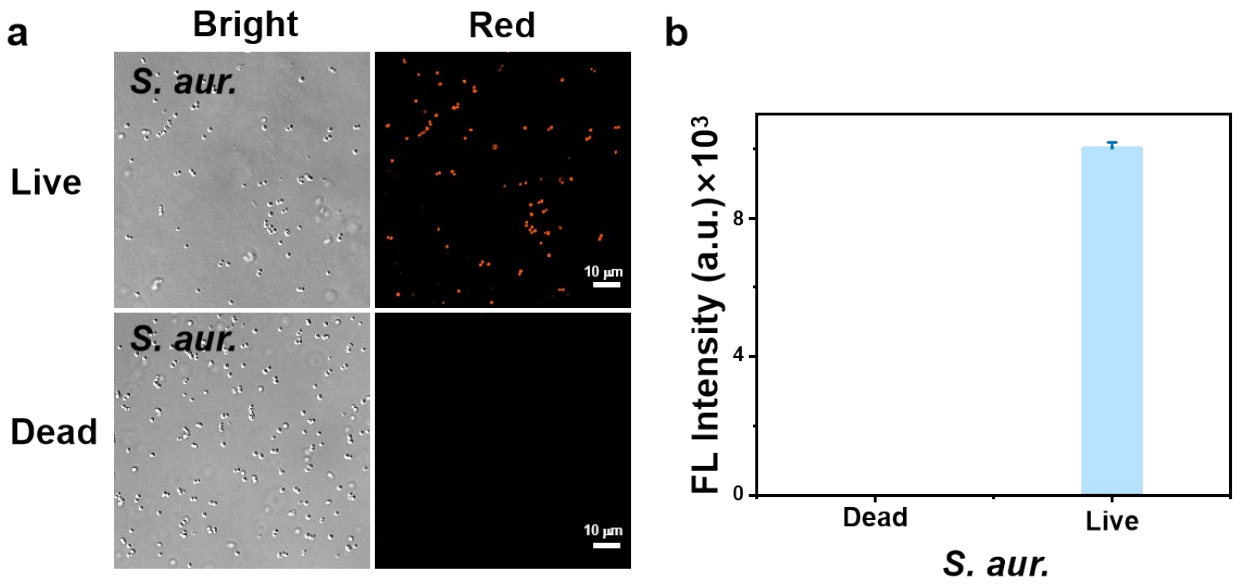

Figure S14. Fluorescence imaging and mean fluorescence intensities of *S. aureus* in normal and dead states incubated with CyQ (5 μM, 4h). Scale bars: 10 μm. λ_{ex} = 533-557 nm, λ_{em} = 570-640 nm. Error bars: standard deviation (SD).

2.10 Cytotoxicity assays on HUVEC cells.

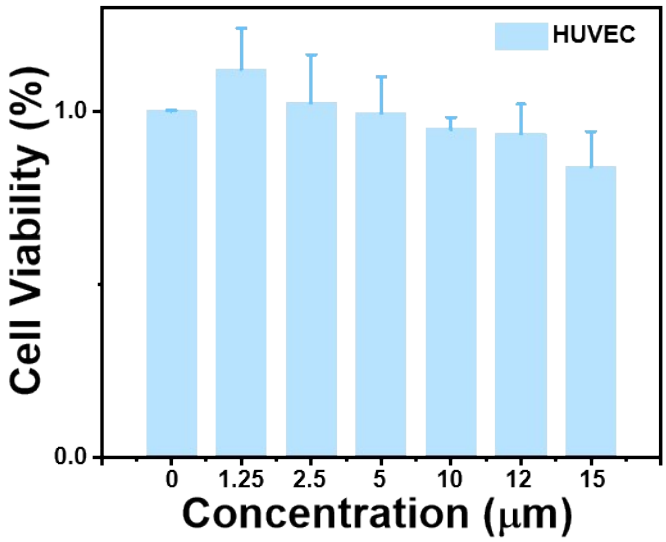

Figure S15. The HUVEC cells viability after incubation with various concentrations of CyQ (0, 1.25, 2.5, 5, 10, 12 and 15 μM) for 24 h. The viability of HUVEC cells without CyQ is defined as 100%. The results are the mean ± standard deviation of five separate measurements.

2.11 The toxicity test of CyQ on bacteria.

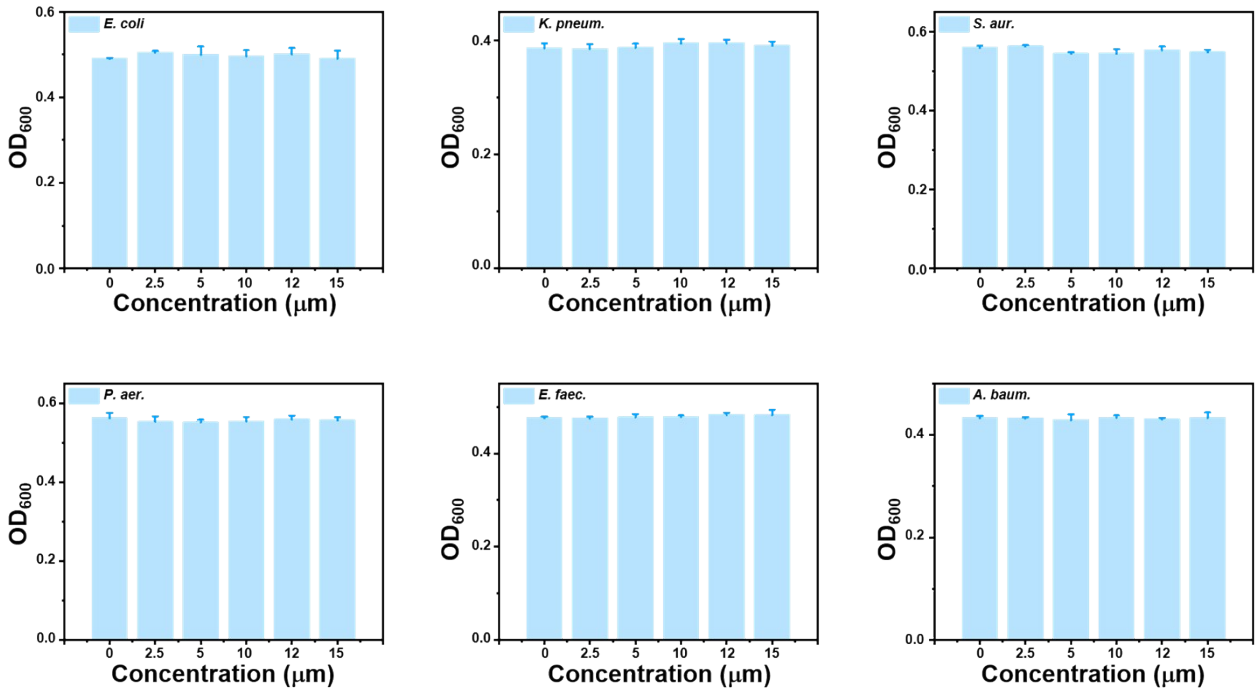

Figure S16. The bacteria (*E. coli, K. pneumoniae, S. aureus, P. aeruginosa, E. faecium and A. baumannii*) viability after incubation with various concentrations of CyQ (0, 2.5, 5, 10, 12, 15 μM) for 12 h. The viability of bacteria without CyQ is defined as 100%. The results represent the mean ± standard deviation of five independent measurements.

2.12 Fluorescence colocalization microscopy analysis of CyQ.

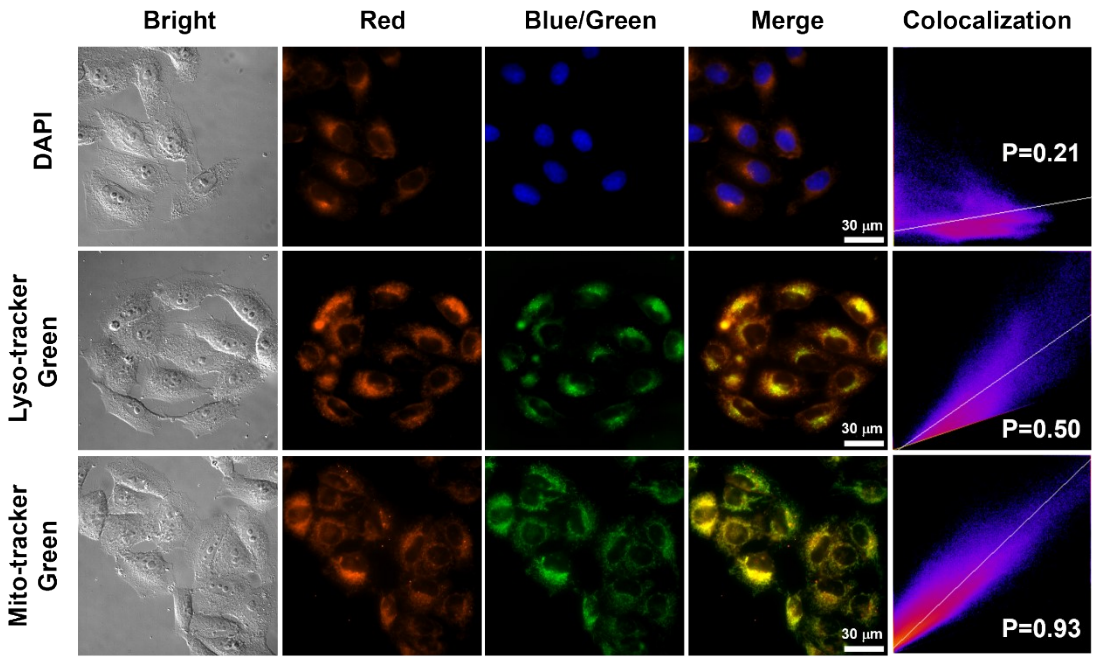

Figure S17. Fluorescence microscopic images of HUVEC cells stained with probe CyQ (5 μM, 2 h) and Lyso-Tracker green (50 nM, 30 min), DAPI (5 μg/ml, 15 min), Mito-Tracker green (200 nM, 15 min). Red channel, λ_{ex}: 533-557 nm, λ_{em}: 570-640 nm, green channel, λ_{ex}: 465-495 nm, λ_{em}: 512-588 nm, blue channel, λ_{ex}: 362-396 nm, λ_{em}: 432-480 nm. Scale bar: 30 μm.

2.13 Fluorescence images of HUVEC cells incubated with CyQ and DAPI.

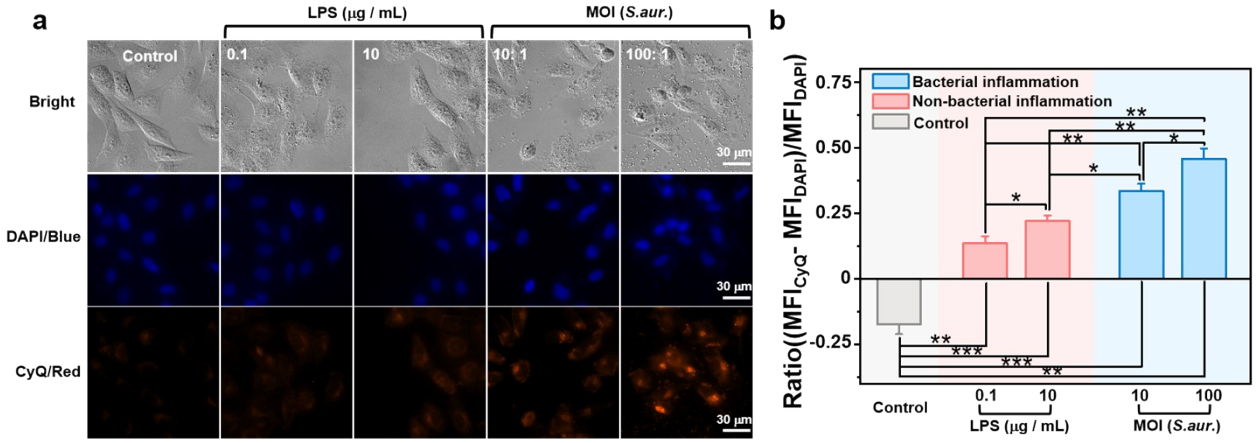

Figure S18. (a) Fluorescence images of HUVEC cells pre-treated with LPS (0.1/10 μg/mL, 24 h), different *S.aureus* MOI (10/100: 1, 3 h) and further incubated with CyQ (5 μM) for 2 h. Scale bars: 30 μm. (b) Summarized data on the ratio value ((mean fluorescent intensities $(MFI)_{CyQ}$ - MFI_{DAPI})/MFI_{DAPI}) of HUVEC cells with the aforementioned treatments. Cell images in the blue channel were acquired with λ_{ex}: 362-396 nm, λ_{em}: 432-480 nm. Cell images in the red channel were acquired with λ_{ex}: 533-557 nm, λ_{em}: 570-640 nm. Error bars: standard deviation (SD). (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).