Supporting Information for

Artificial protein-probe hybrid as a responsive probe for ratiometric detection and imaging of hydrogen peroxide in cells

Xing Zhang,[a,b] *Youxin Fu,* [b] *Guangren Qian,**, [a] *Run Zhang,* [b] *and Zhi Ping Xu**,[b]

[a] School of Environmental Science and Chemical Engineering, Shanghai University, Shanghai 200444, China

[b] Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St Lucia, QLD 4072, Australia

Table of Contents

P1. General Information

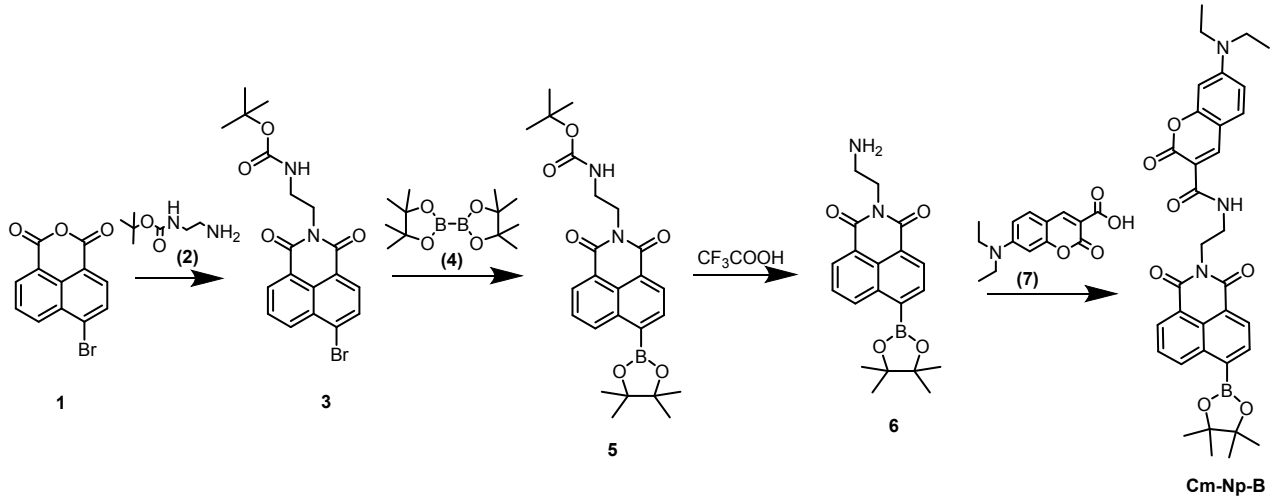
Materials and Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lipopolysaccharide (LPS), were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), *L*-glutamine, penicillin, and streptomycin sulfate were purchased from Life Technologies. The PBS buffer consisting of 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 was prepared in our laboratory. Chemicals were used as received unless otherwise indicated. All oxygen or moisture sensitive reactions were performed under argon atmosphere using the standard Schlenk method. All other reagents are of analytical purity and used without further treatment. Solvents used are of analytical grade, except those for recrystallization and optical tests, which were distilled prior to use.

Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with silica gel 60 F254 (MERCK). ^{1}H NMR and ^{13}C NMR Spectra were recorded using Bruker AM-400 Spectrometers. High resolution mass spectra (HRMS) were recorded on a Xevo G2-XS QTof Quadrupole Time-of-Flight Mass Spectrometry. DMSO-d_6, $CDCl_3$ were used as solvent. Absorption and fluorescence spectra were recorded using Varian Cary 500 and Varian Cary Eclipse, respectively. Dynamic light scattering of size distribution was measured on Nano Zeta-Sizer (Malvern Instruments). Confocal fluorescence imaging in live cells were carried out on a Leica SP8 laser-scanning microscope (λ_{ex} = 405, λ_{em} = 450-480 nm (blue channel), 520-550 nm (green channel)). The images were analysis by ImageJ software version 1.44p. Flow cytometry analysis was performed on an Accuri C6 flow cytometer with a 405 nm laser excitation and emission filter of 450 ± 20 nm and 525 ± 20 nm for blue and green channel, respectively. The data were analyzed with CytExpert software. All data were presented as the mean ± SD for each group of three experiments.

Synthesis of Cm-Np-B.

The procedure for the synthesis of **Cm-Np-B** was presented in Scheme S1 and the details for the synthesis and structural characterization of **Cm-Np-B** were described in the following.



Scheme S1. Synthesis procedure of **Cm-Np-B** .

Synthesis of Compound 3: Compound **1** (1.50 g, 5.4 mmol) and compound **2** (2.60 g, 16.2 mmol) in a 20 mL ethanol solution was stirred and heated at 75 °C for 4 h. The reaction was monitored by TLC. After cooling down to room temperature, the resulting mixture was concentrated by a rotary evaporator in vacuum. The residue was purified by column chromatography on silica gel using dichloromethane/ethyl acetate (6:1, v/v) as the eluent to afford compound **3** (2.10 g, 90% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.65 (dd, J = 7.3, 0.9 Hz, 1H), 8.56 (dd, J = 8.5, 1.0 Hz, 1H), 8.41 (d, J = 7.9 Hz, 1H), 8.03 (d, J = 7.9 Hz, 1H), 7.87 – 7.81 (m, 1H), 4.35 (t, J = 5.6 Hz, 2H), 3.59 – 3.46 (m, 2H), 1.27 (s, 9H).

Synthesis of Compound 5: Under nitrogen atmosphere, a mixture of compound **3** (0.65 g, 1.56 mmol), bis(pinacolato)diboron (0.52 g, 2.34 mmol), $Pd(dppf)Cl_2$ (34 mg, 0.08 mmol) and potassium acetate (0.46 g, 4.68 mmol) in dioxane (15 mL) was stirred and heated at 90 °C overnight. The reaction was monitored by TLC. After cooling down to room temperature, the reaction mixture was

diluted with CH_2Cl_2, washed with H_2O and brine, and dried by Mg_2SO_4. The resulting residue was purified by column chromatography on silica gel using petroleum ether/CH_2Cl_2 (2:1, v/v) as the eluent to afford compound **5** (0.51 g, 70% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.12 (dd, *J* = 8.4, 0.7 Hz, 1H), 8.64 – 8.55 (m, 2H), 8.30 (d, *J* = 7.3 Hz, 1H), 7.82 – 7.74 (m, 1H), 5.30 (s, 1H), 4.37 (t, *J* = 5.4 Hz, 2H), 3.58 – 3.49 (m, 2H), 1.46 (s, 12H), 1.29 (s, 9H).

Synthesis of Compound Cm-Np-B: Compound **5** (0.26 g, 0.57 mmol) was dissolved in 10 mL TFA/CH_2Cl_2 (1:4, v/v) and the mixture was stirred at room temperature for 2 h. The solvent was evaporated to afford yellowish compound **6** (0.21 g). The compound **6** was used directly to the next phase without further purification.

Under N_2 atmosphere, compound **7** (0.18 g, 0.70 mmol), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 0.26 g, 2 mmol) and 1-Hydroxybenzotriazole (HOBt, 0.38 g, 2 mmol) were mixed and stirred in anhydrous DMF at 0 °C for 30 min. Compound **6** (0.21 g, 0.57 mmol), triethylamine (0.30 mL) were sequentially added. The mixture was then stirred at room temperature for 24 h and the reaction was monitored by TLC. The reaction mixture was poured into water and filtered to yield yellowish powder. This crude product was purified by column chromatography on silica using CH_2Cl_2/methanol (10:1, v/v) as the eluent to yield compound **Cm-Np-B** (0.21 g, 50% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.95 (d, *J* = 8.4 Hz, 1H), 8.73 (t, *J* = 5.6 Hz, 1H), 8.47 (d, *J* = 6.7 Hz, 1H), 8.41 (t, *J* = 6.6 Hz, 2H), 8.17 (d, *J* = 7.2 Hz, 1H), 7.84 (dd, *J* = 15.9, 8.1 Hz, 1H), 7.56 (d, *J* = 8.9 Hz, 1H), 6.72 (d, *J* = 8.6 Hz, 1H), 6.52 (s, 1H), 3.64 (d, *J* = 5.4 Hz, 4H), 3.43 (dd, *J* = 13.4, 6.5 Hz, 4H), 1.38 (s, 12H), 1.10 (t, *J* = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.64 (s), 165.75 (s), 164.37 (s), 160.22 (s), 155.43 (s), 150.59 (s), 138.66 (s), 137.56 (s), 137.12 (s), 134.56 (s), 133.40 (s), 132.32 (s), 130.44 (d, *J* = 17.0 Hz), 127.60 (s), 125.55 (s), 113.11 (s), 112.52 (s), 110.60 (s), 98.89 (s), 87.56 (s), 58.00 (s), 47.41 (s), 40.49 (s), 27.80 (s), 15.39

(s). TOF MS ES$^+$ m/z: [M + H]$^+$ calcd. 632.2544, found 632.2537.

Preparation of protein-probe hybrid Cm-Np-B@BSA

Stock solutions of **Cm-Np-B** was prepared in DMSO (1 mM), which were then transferred to 10 mL volumetric flasks, BSA (1 mM) was prepared in phosphate buffered saline (PBS, 0.01 M, pH 7.4). **Cm-Np-B@BSA** (5/50 μM) hybrid solution was prepared in the following three steps.

1. 100 μL BSA solution was added to 1.9 mL PBS buffer solution under vigorous stirring;
2. 10 μL **Cm-Np-B** solution was slowly added to the mixture under vigorous stirring;
3. The resulting mixture was incubated for 5 min to allow for sufficient host-guest binding.

The binding of Cm-Np-B to BSA was then confirmed by fluorescence spectroscopy and Dynamic Light Scattering (DLS).

General procedure for fluorescence spectrometric analysis

For spectrometric analysis of the binding between **Cm-Np-B** and BSA: the **Cm-Np-B** in DMSO was added into the PBS buffer containing increased concentration of BSA under vigorous stirring. The mixture was then stirred at R. T. for another 5 min, and then was subjected to fluorescence spectrometric analysis.

For spectrometric analysis of H_2O_2: for the solution of protein-probe hybrid **Cm-Np-B@BSA**, H_2O_2 at different concentration was added into the solution. The mixture was stirred at R. T. for 10 min, and then the fluorescence spectra were recorded (λ_{ex} = 410 nm).

Dynamic Light Scattering (DLS)

The size of BSA, **Cm-Np-B**, and **Cm-Np-B@BSA** were measured on a Nano Zeta-Sizer (Malvern Instruments). The concentration of these three samples are 50, 5, and 5/50 μM, respectively. All the experiments were measured in PBS buffer at 298K.

H_2O_2 Detection in Solution Using Cm-Np-B@BSA as a Probe

For titration experiments, H_2O_2 (0-200 μM, interval: 10 μM) was added to solution of **Cm-Np-B@BSA**. After incubation at room temperature for 5 min, the fluorescence spectra of each solution were measured. For selectivity measurements, various reactive oxygen species, anions and biomolecules (500 μM) were added into the solution of **Cm-Np-B@BSA**. The mixture was then incubated at room temperature for 5 min, and then the fluorescence spectra were recorded. The excitation wavelength was at 410 nm and emission between 425 nm and 700 nm were recorded. The excitation and emission slits were 5 nm.

pH dependence experiment for H_2O_2 Detection

For pH dependence experiments, **Cm-Np-B@BSA** was added to different pH solution (pH 5, 6, 7.4, 8), and then the fluorescence spectra of each solution were measured. Then, H_2O_2 (200 μM) was added to these solutions, which were incubated at room temperature for 10 min before subjecting to fluorescence spectrum analysis. The excitation wavelength was at 410 nm and emission between 425 nm and 700 nm was recorded. The excitation and emission slits were 5 nm.

Cells and Cell Culture

RAW 264.7 microphage cells (ATCC®TIB-71™) was obtained from American Type Cell Collection. The cells were incubated in Dulbecco's Modified Eagle's Medium, which was supplemented with 10% fetal bovine serum and penicillin (10 U/mL)/streptomycin (10 μg/mL). All cells were cultured in a humidified incubator at 37 °C and 5% CO_2. The cell growth medium was replaced every two days. RAW 264.7 cells were routinely detached with trypsin-EDTA solution and then seeded in a 25 mL cell culture bottle. The cells were reached about 80% confluence before experiments.

Fluorescence Imaging of H_2O_2 in Live Cells

For fluorescence imaging of H_2O_2, RAW 264.7 macrophage cells were typically seeded at a density of 5×10^4 cells/mL in a cover glass-bottomed cell culture dish (ϕ = 20 mm). The cells were supplied

with DMEM buffer and cultured in an incubator at 37 °C for 24 h. The cell culture medium was replaced with freshly prepared medium containing **Cm-Np-B@BSA**, and then the cells were incubated at 37 °C for another 2 h. After washing with PBS for three times, the **Cm-Np-B@BSA**-internalized, cells were treated with H_2O_2 (200 μM, 30 min at 37 °C). Then, the cells were subjected to confocal microscopy fluorescence imaging. The **Cm-Np-B@BSA**-internalized cells were used as the control group.

Flow Cytometry of H_2O_2 in Live Cells

RAW 264.7 cells were seeded in a 6-well plate at a density of 1×10^6 cells/well. After incubating for 24 h, the cell culture medium was replaced with 1 mL of fresh medium containing **Cm-Np-B@BSA**. The cells were then incubated in an incubator at 37 °C and 5% CO_2 for another 2 h. The culture medium was removed and then the cells were treated with H_2O_2 (200 μM) for 2 h. The macrophage cells were then detached from well with cold PBS. The cells were collected, washed with PBS and tested under cytometer.

MTT Cytotoxicity Assay

The cytotoxicity of **Cm-Np-B@BSA** toward RAW 264.7 macrophage cells was assessed by the typical 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Typically, cells in growth medium were seeded in a 96-well plate at a density of 5000 cells per well. After 24 h incubation, the culture medium was then replaced with a fresh medium containing **Np-Cm-B@BSA** of different concentrations. After further incubation in a humidified incubator for adherence for 24 h, MTT diluted by DMEM (FBS free) medium (10%) was added to each well after the removal of culture media and incubated for 4 h in a dark room at 37 °C. The excess MTT medium was removed and dimethyl sulfoxide (DMSO, 100 μL) was added to each well to dissolve the MTT formazan for 10 min. The absorbance of converted dye in DMSO was measured at a wavelength of 490 nm. The

results from the five individual experiments were averaged. The following formula was used to calculate the viability of cell growth:

Viability (%) = (mean of absorbance value of treatment group-blank)/(mean absorbance value of control-blank) × 100.

P2. NMR and MS Spectra of Cm-Np-B and Intermediates

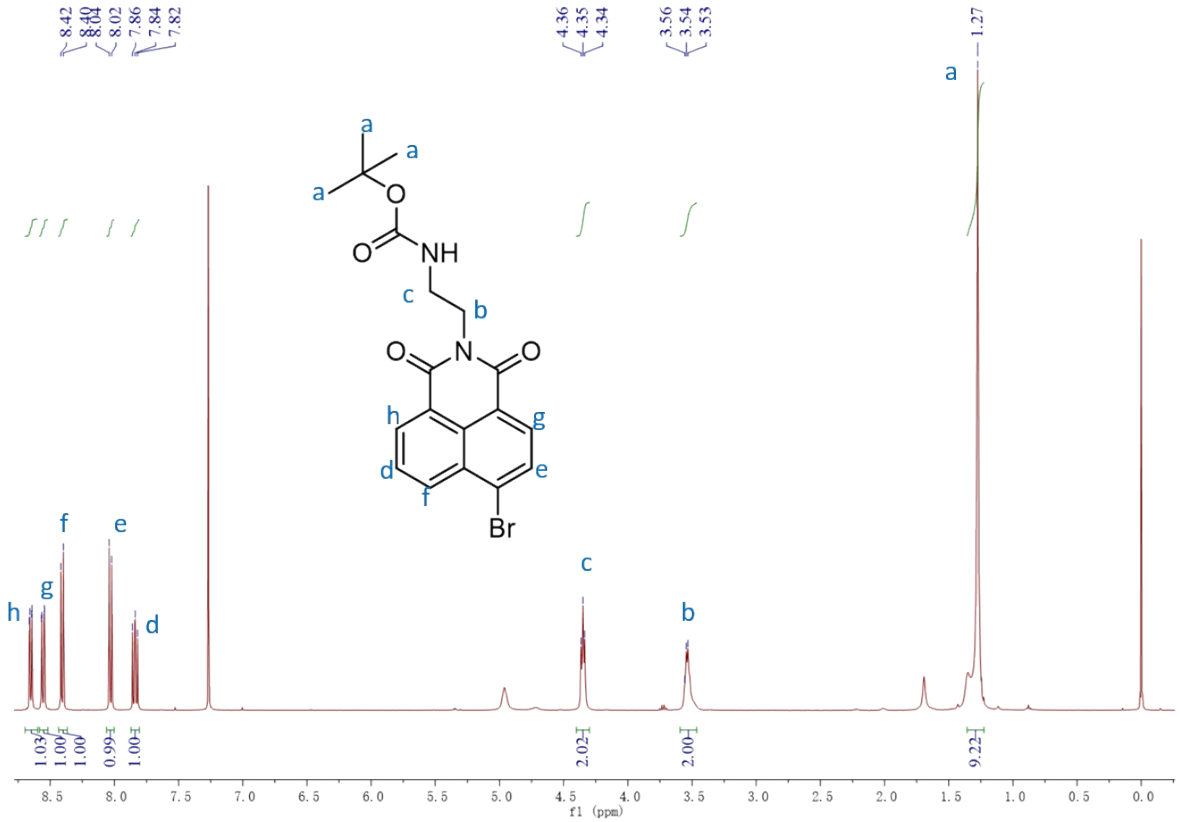


Fig. S1. ^{1}H NMR of **Compound 3.**

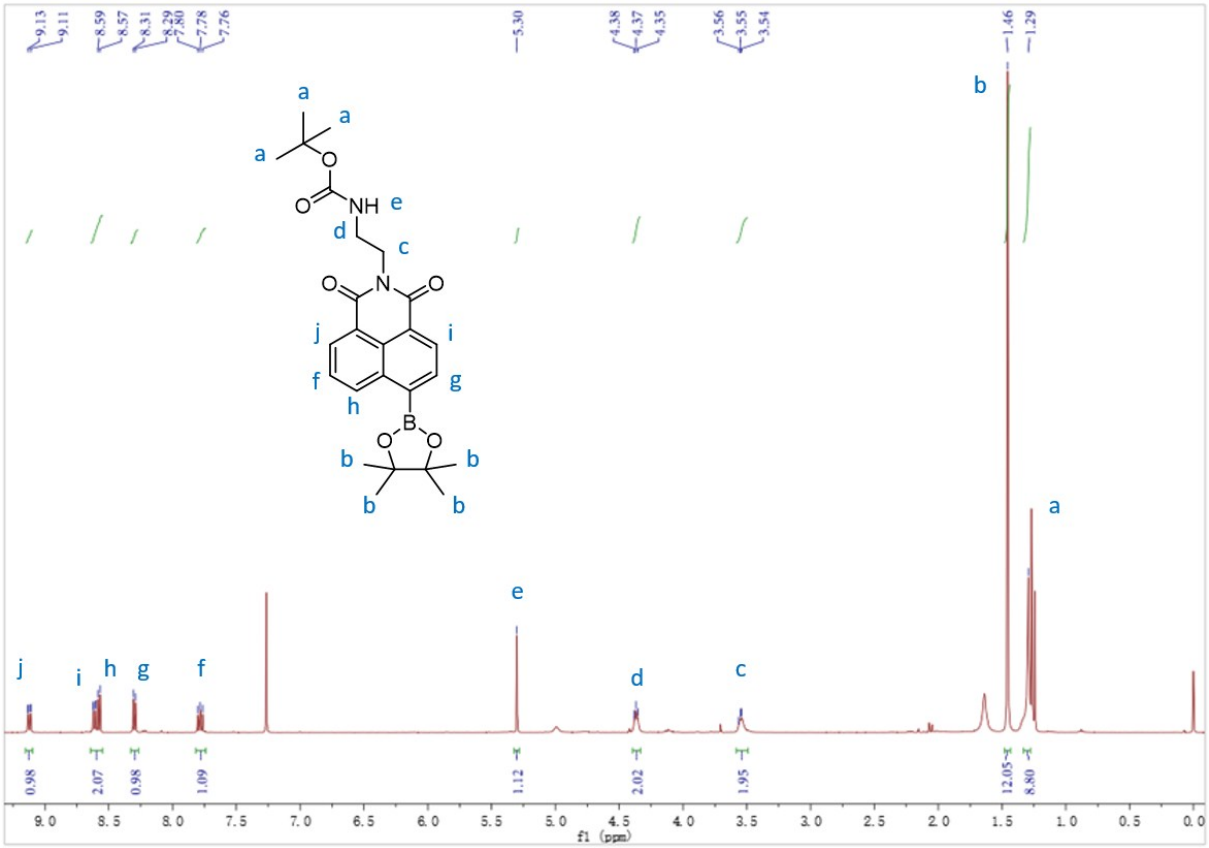


Fig. S2. ^{1}H NMR of **Compound 5.**

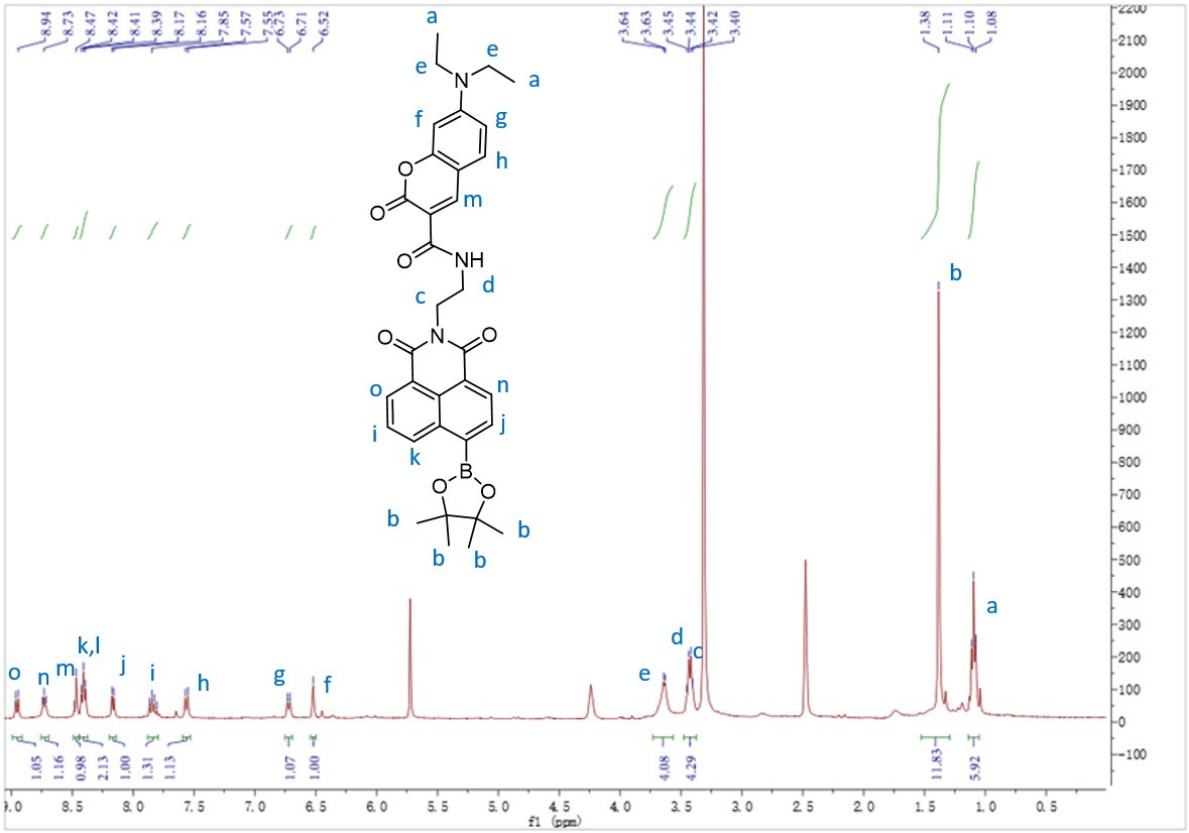


Fig. S3. [1]H NMR of Compound **Cm-Np-B.**

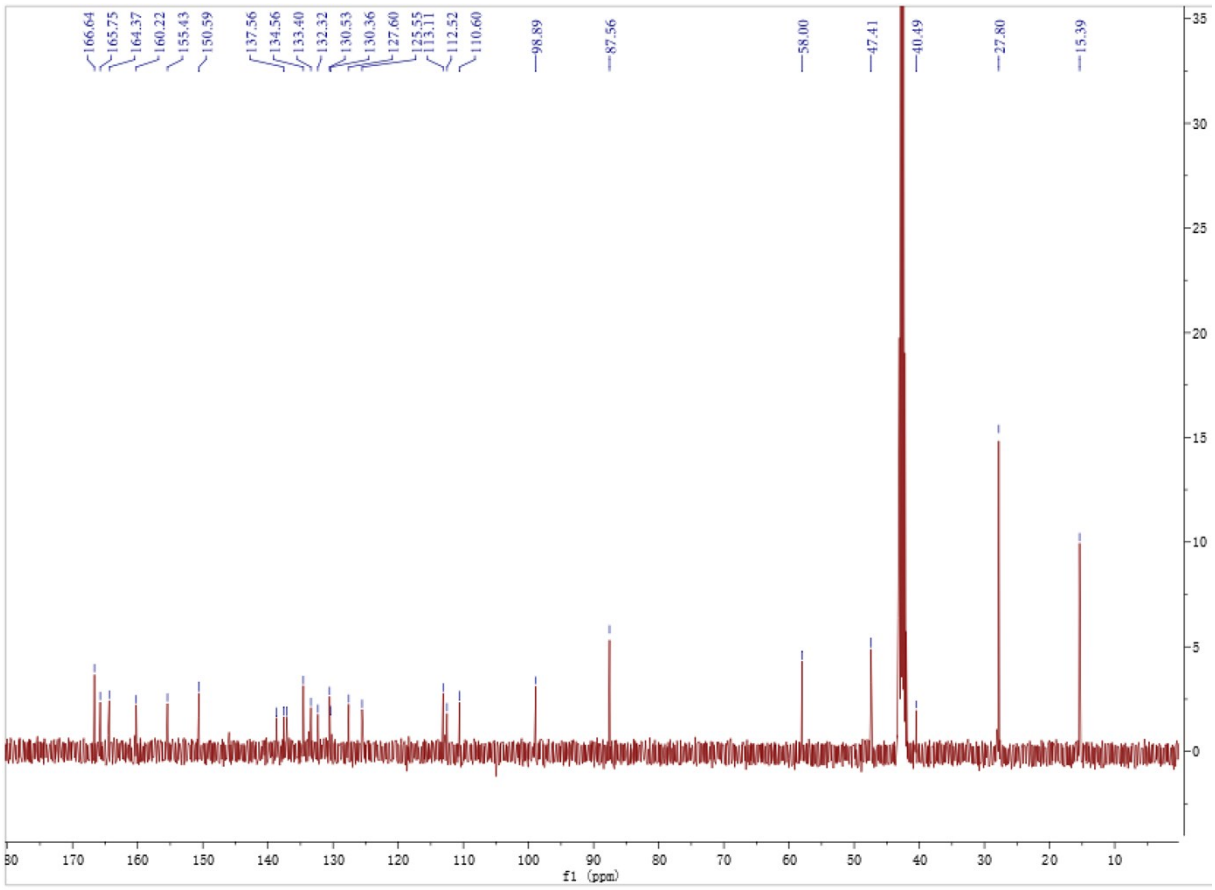


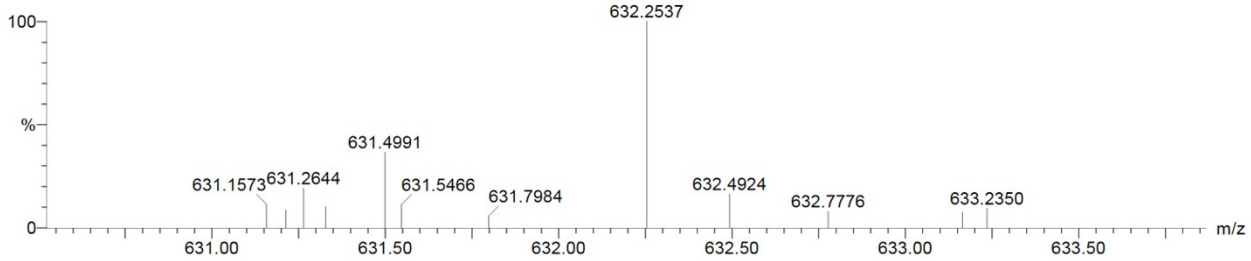
Fig. S4. ^{13}C NMR of Compound **Cm-Np-B.**

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
110 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 0-34 H: 0-36 N: 0-3 O: 0-7 B: 0-1 Na: 0-1

H-TIAN
TH-WWH-101 129 (1.466) Cm (122:131) 1: TOF MS ES+
2.03e+002



Minimum:				-1.5			
Maximum:		5.0	20.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
632.2537	632.2544	-0.7	-1.1	18.5	33.3	0.0	C34 H36 N3 O7 B Na

Fig. S5. TOF MS ES+ of Compound **Cm-Np-B.**

P3. Additional figures and tables

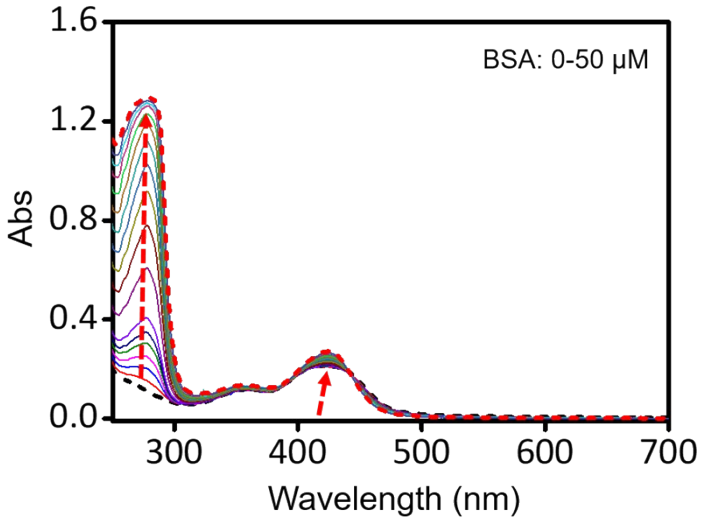


Fig. S6. UV-Vis absorption spectra of **Cm-Np-B** (5 μM) with different concentrations of BSA (0-50 μM). All measurements were carried out in PBS buffer (containing 0.5% DMSO (v/v), pH 7.4).

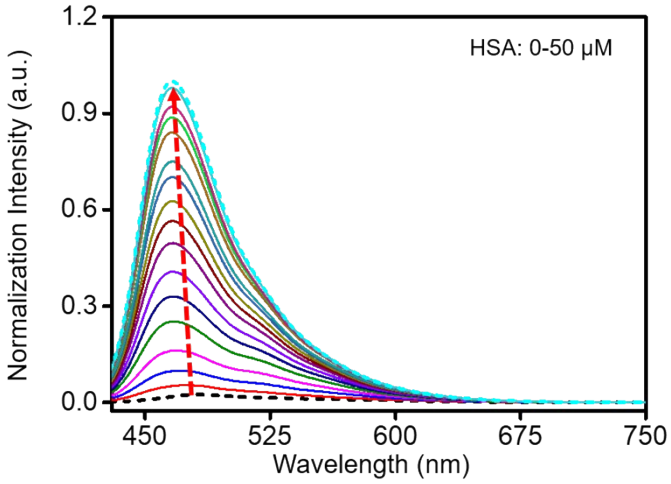


Fig. S7. Fluorescence spectra of **Cm-Np-B** (5 μM) with different concentrations of HSA (0-50 μM). All measurements were carried out in PBS buffer (containing 0.5% DMSO (v/v), pH 7.4).

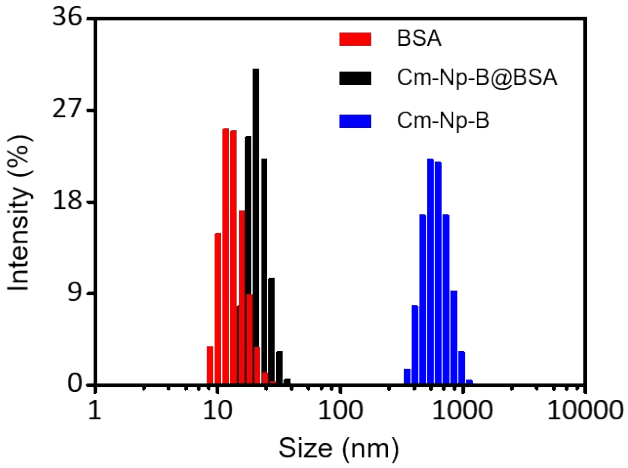


Fig. S8. Dynamic light scattering of **BSA** (red)**, Cm-Np-B** (blue) and **Cm-Np-B @BSA** (black).

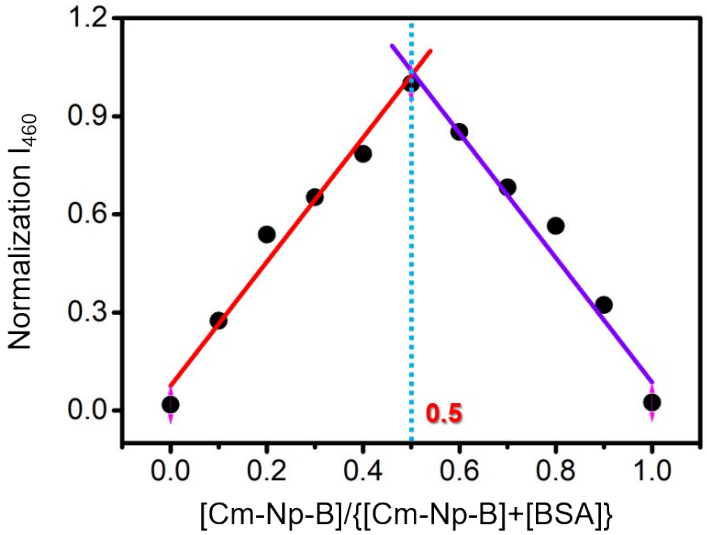


Fig. S9. Fluorescence Job's plot for the analysis of **Cm-Np-B@BSA** binding ratio.

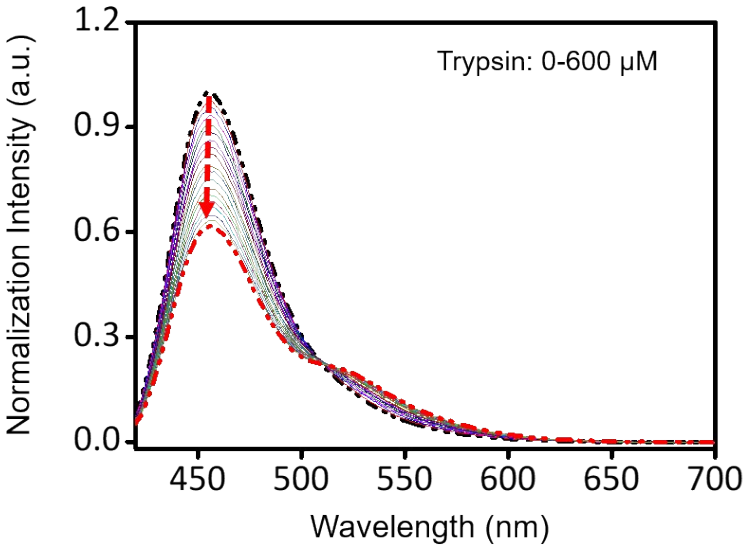


Fig. S10. Fluorescence intensity change of **Cm-Np-B@BSA** (5/50 μM) with different concentrations of Trypsin (0-600 μM). All measurements were carried out in PBS buffer (containing 0.5% DMSO (v/v), pH 7.4, excitation wavelength: 410 nm).

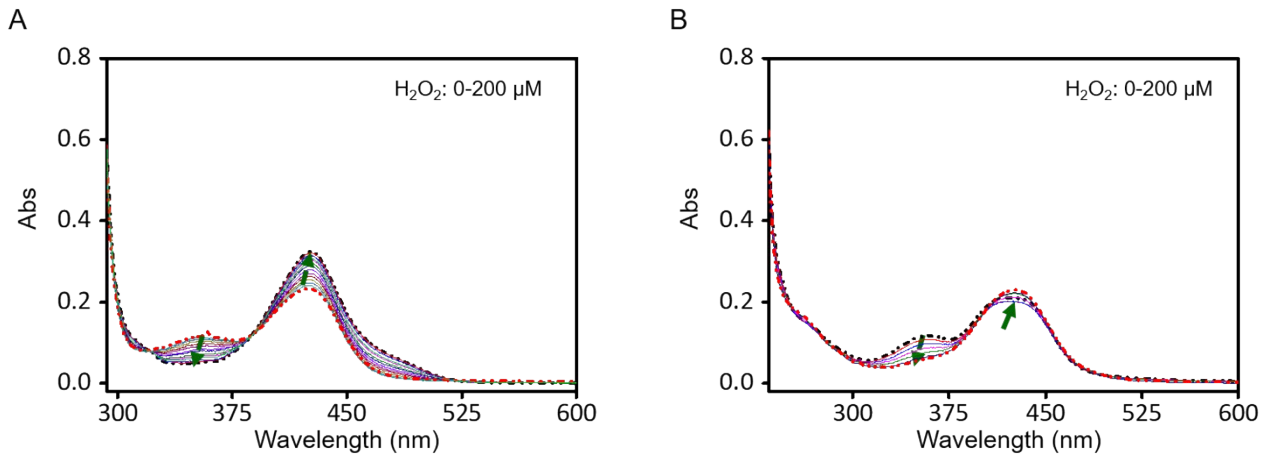


Fig. S11. UV-Vis absorption spectra of (A) **Cm-Np-B@BSA** (5/50 μM) and (B) **Cm-Np-B** (5 μM) with different concentrations of H_2O_2 (0-200 μM). All measurements were carried out in PBS buffer (containing 0.5% DMSO (v/v), pH 7.4).

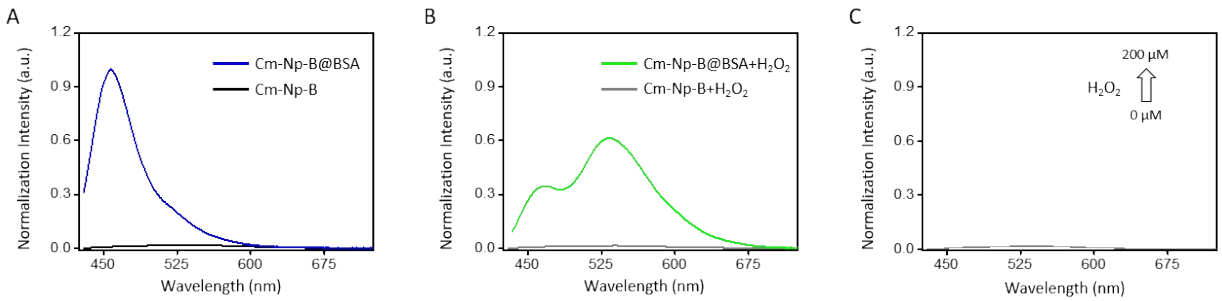


Fig. S12. A) Fluorescence spectra of Cm-Np-B@BSA(5/50 μM) and Cm-Np-B (5 μM); B) fluorescence spectra of Cm-Np-B@BSA(5/50 μM) and Cm-Np-B (5 μM) with H_2O_2 (200 μM); and C) fluorescence response of probe Cm-Np-B (5 μM) with H_2O_2 (0-200 μM). All these experiments were tested in PBS buffer.

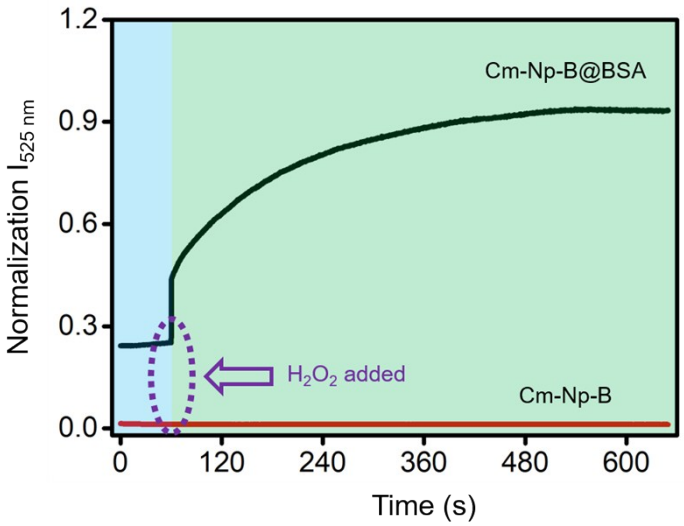


Fig. S13. Time-dependent fluorescence response of probe **Cm-Np-B@BSA** (5/ 50 μM, black line) and **Cm-Np-B** (5 μM, red line) toward H_2O_2 (200 μM) in PBS buffer (0.5% DMSO, pH 7.4).

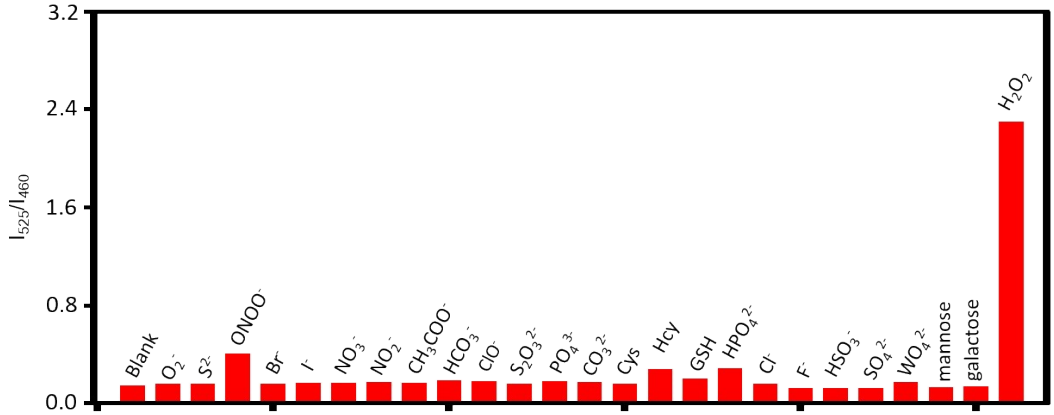


Fig. S14. Fluorescence intensity change of **Cm-Np-B@BSA** (5/50 μM) in the presence of various interfering analytes in 10 min. Blank (**Cm-Np-B@BSA**), O_2^-, S^{2-}; $ONOO^-$; Br^-; I^-; NO_3^-; NO_2^-; CH_3COO^-; HCO_3^-; ClO^-; $S_2O_3^{2-}$; PO_4^{3-}; CO_3^{2-}; Cys; Hcy; GSH; HPO_4^{2-}; Cl^-; F^-; HSO_3^-; SO_4^{2-}; WO_4^{2-}; mannose; galactose; H_2O_2, respectively. H_2O_2: 200 μM; other species: 500 μM. All measurements were carried out in PBS buffer, pH 7.4, excitation wavelength: 410 nm).

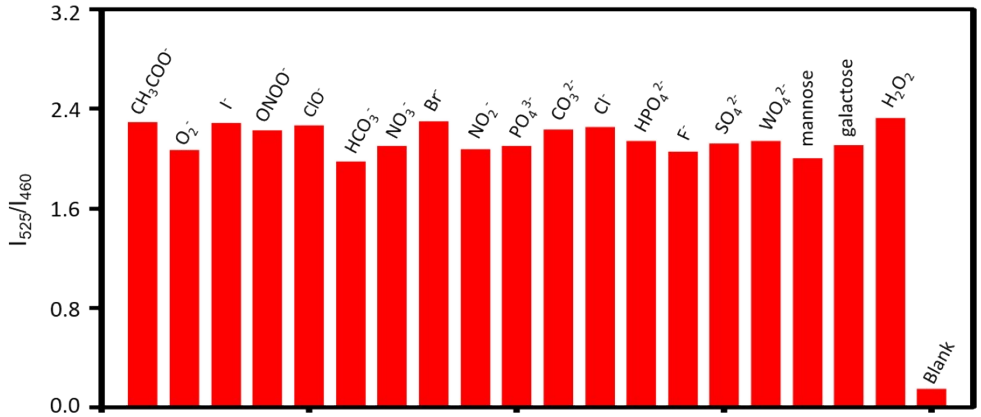


Fig. S15. Competitive selectivity experiment of **Cm-Np-B@BSA** (5/50 μM) in the presence of various interfering "analytes+H_2O_2" in 10 min. All measurements were carried out in PBS buffer, pH 7.4, excitation wavelength: 410 nm).

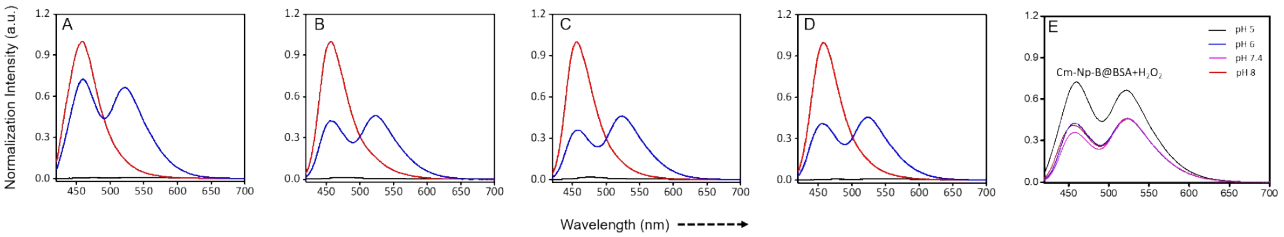


Fig. S16. Fluorescence Intensity changes of **Cm-Np-B@BSA** (5/50 μM) with or without H_2O_2 in different pH of PBS buffer solution (0.5% DMSO (v/v), A) pH 5; B) pH 6; C) pH 7.4; D) pH 8; black line, red line and blue line represent **Cm-Np-B**, **Cm-Np-B@BSA** and **Cm-Np-B@BSA+H_2O_2** respectively; E) fluorescence intensity of **Cm-Np-B@BSA+H_2O_2** in different pH solutions with the excitation wavelength at 410 nm.

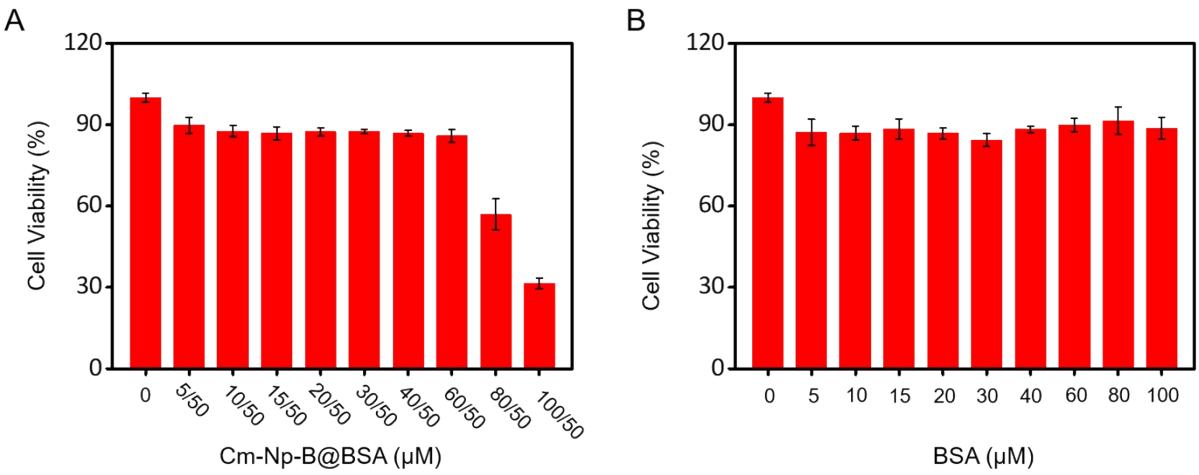


Fig. S17. MTT cytotoxicity assay. Viability of Raw 264.7 cell in the presence of different concentration **Cm-Np-B@BSA** (A) and BSA only (B).

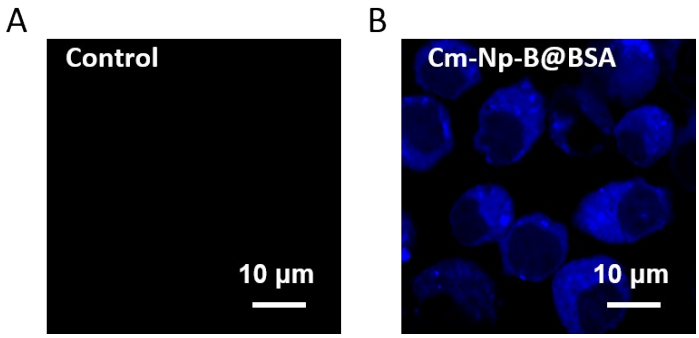


Fig. S18. Fluorescence imaging of control group (A) and **Cm-Np-B@BSA** (B) in Raw 264.7 Cell. The excitation wavelength was 405 nm and emission channel was 460-480 nm. The images were visualized by confocal laser-scanning microscopy (scale bar = 10 μm).

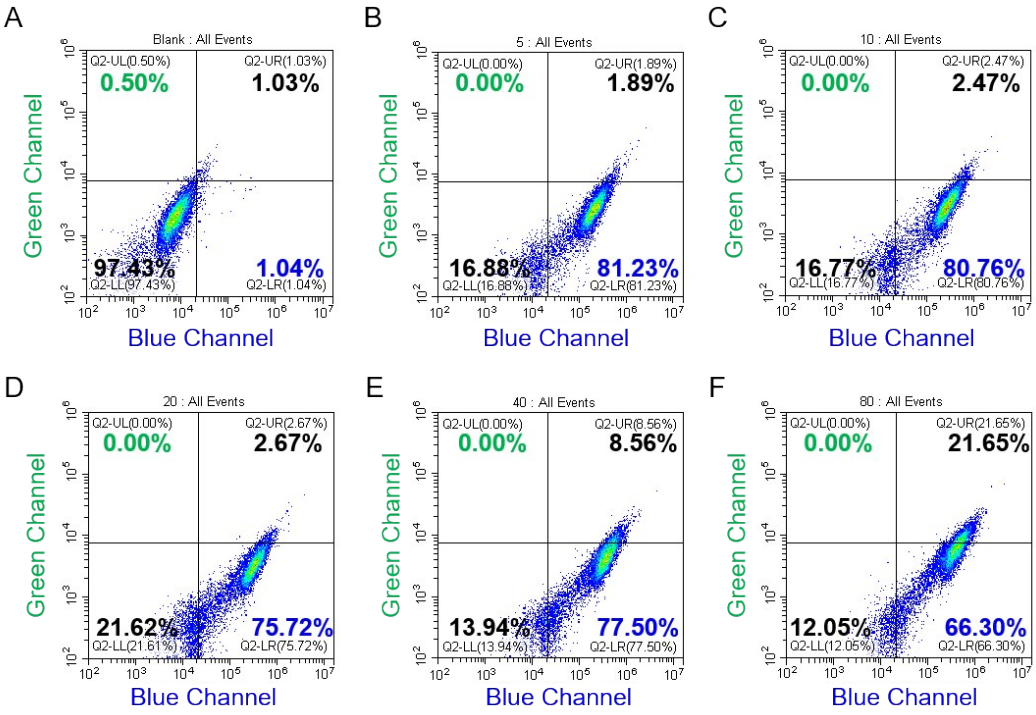


Fig. S19. Flow Cytometric analysis of different concentration **Cm-Np-B@BSA** in Raw 264.7 Cell.

From A to F: Blank; 5/25 μM; 5/50 μM; 5/100 μM; 5/200 μM; 5/400 μM respectively.

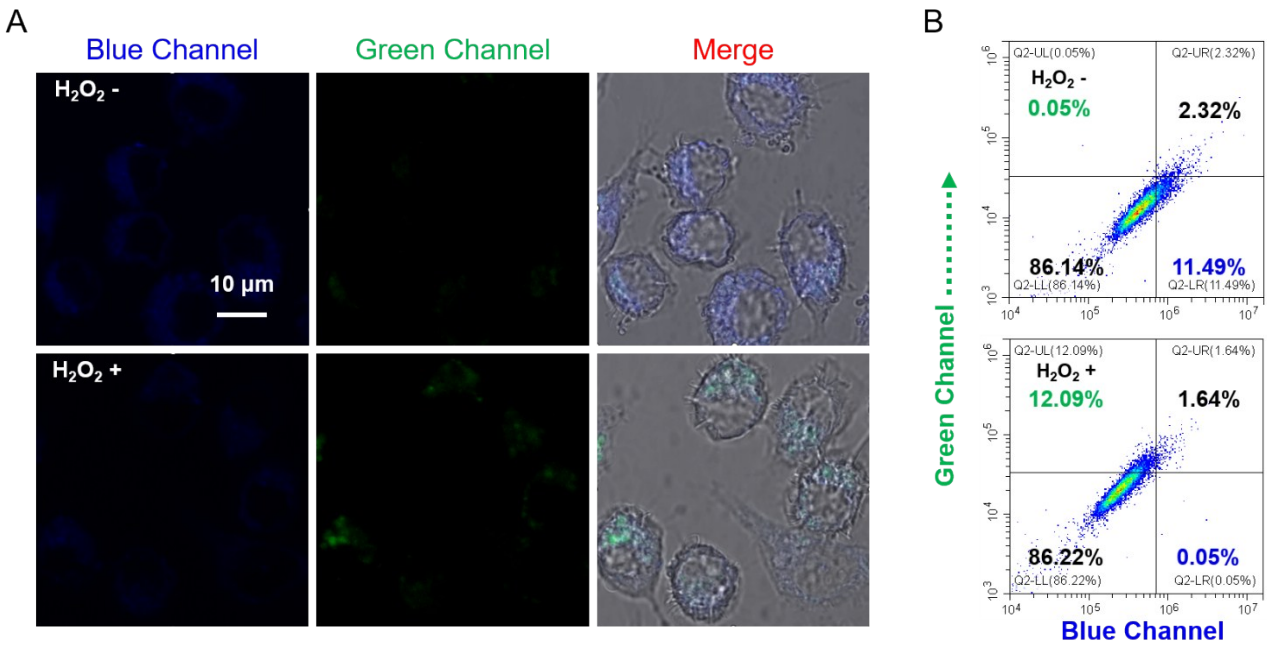


Fig. S20. (A) Fluorescence imaging of **Cm-Np-B** (5 μM) with (H_2O_2+) or without (H_2O_2-) exogenous H_2O_2 (200 μM) in Raw 264.7 Cell. (B) Flow Cytometric analysis of **Cm-Np-B** (5 μM) with (H_2O_2+) or without (H_2O_2-) exogenous H_2O_2 (200 μM) in Raw 264.7 Cell. The excitation wavelength was 405 nm and emission channel 460-480 nm, 520-560 nm for Blue, Green Channel, respectively. The images were visualized by confocal laser-scanning microscopy (scale bar = 10 μm).