Supporting information for

Chemically Engineered Water-Soluble Block Copolymer for Redox Responsive SO_2 Release in Antibacterial Therapy

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Experimental section

Materials. 2,4-Dinitrobenze sulfonyl chloride (2,4-DNSCl, 98%, Alfa Aesar), ethanol amine (98%, Sigma), methacrylic acid (Sigma), triethyl amine (Et_3N, Sigma), dicyclohexylcarbodiimide (DCC, 99%, Sigma), 4-dimethylamino pyridine (DMAP, 99%, Sigma), sodium carbonate (Na_2CO_3, Merck), sodium bisulfite ($NaHSO_3$, Sigma), aluminium oxide (Sisco Research Laboratories (SRL) Pvt. Ltd., India), sodium chloride (NaCl, Merck), magnesium sulfate ($MgSO_4$, Merck), L-glutathione reduced (GSH, 98%, Sigma), Nile red (98%, Sigma) were used as received. Ethanol, glacial acetic acid, hydrochloric acid, anhydrous *N*,*N*′- dimethylformamide (DMF, 99.9%), and phosphate buffer saline (PBS, 10X) were bought from SRL. Polyethylene glycol methyl ether methacrylate (PEGMA, average molar mass = 300 g/mol) was purchased from Sigma and purified through a basic alumina column prior to use. 2,2′-Azobis(2-methylpropionitrile) (AIBN, 98%, Sigma) was purified by crystallization in methanol twice before using as an initiator in the polymerization reactions.

4-Cyano-(dodecylsulfanylthiocarbonyl)sulfanylpentanoic acid (CDP) was synthesized according to the previous literature procedure.[1] 7-Diethylaminocoumarin-3-aldehyde (DEACA) was synthesized following the previous literature method.[2] For NMR spectroscopic measurements, chloroform-*d* ($CDCl_3$, 99.8% D), dimethyl sulfoxide-d_6 (DMSO-d_6, 99.9% D), and deuterium oxide (D_2O, 99% D) were obtained from Cambridge Isotope Laboratories, Inc. (USA). Hexane (mixture of isomers), dichloromethane (DCM), ethyl acetate, acetone, methanol, and diethyl ether were purchased from Merck (India) and used without further purifications. Agar-agar, type I, Mueller Hinton (MH) broth, and Luria broth (LB), Dulbecco's modified Eagle medium (DMEM), Hank's balanced salt solution (HBSS) and fetal bovine serum were acquired from HiMedia. Millipore Milli-Q grade water was used in all experiments. Thiazolyl blue tetrazolium bromide (MTT, extrapure AR, 98%), 2,7-dichlorofluorescein diacetate (extrapure, 97%, DCFDA), dimethyl sulfoxide (DMSO, 99.8%), Hoechst 33342, propidium iodide (PI) and 10% Titrion X were purchased from SRL. Bacteria strains *Bacillus subtilis*, and *Escherichia coli* were obtained from MTCC. HeLa Kyoto cell line was acquired from ATCC. For purification of polymers, dialysis membranes (Spectra/Por® 7, molecular weight cut-off (MWCO): 2000 Da) were obtained from Spectrum Laboratories, Inc.

Instruments and characterizations. *Nuclear magnetic resonance (NMR)*. The ^{1}H and ^{13}C NMR spectra were performed using either 400 MHz Bruker or 500 MHz Bruker Avance^{III} NMR spectrometer at room temperature.

Attenuated total reflection Fourier transform infrared (ATR-FTIR). The FTIR spectra were collected in solid state in a transmission mode on a Perkin-Elmer Spectrum 2 spectrometer (L160000A).

Size exclusion chromatography (SEC). Size exclusion chromatography (SEC) was performed to obtain the molar mass ($M_{n,SEC}$) and dispersity ($Đ$) of polymers in DMF eluent with a 0.8 mL/min flow rate at 40 °C. The instrument contains a Waters 1515 HPLC pump, Waters 2414 refractive index (RI) detector, two PolarGel-M analytical columns (300 × 7.5 mm), and one PolarGel-M guard column (50 × 7.5 mm). Poly(methyl methacrylate) (PMMA) standards were used to calibrate the instrument.

UV-Vis spectroscopy. Spectra of all the samples were recorded with a Perkin Elmer Lambda 35 spectrophotometer at 25 ^{o}C.

Fluorescence spectroscopy. All fluorescence spectra were recorded in a Horiba JobinYvon (Fluoromax-3, Xe-150 W, 250-900 nm) fluorescence spectrophotometer.

Dynamic light scattering (DLS). DLS was recorded using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., UK) equipped with a laser beam of He-Ne, functioning at 633 nm at a scattering angle of 173° at room temperature.

Transmission electron microscopy (TEM). The images were obtained using a JEOL JEM-2100F electron microscope functioning at 120 kV.

Field emission scanning electron microscopy (FESEM). The images were recorded using Carl Zeiss Supra 55 VP FESEM.

Confocal laser scanning microscopy (CLSM). Bacteria cell imaging data were recorded with a CARL-ZEISS inverted laser scanning confocal microscope (LSM880).

Synthesis of *N*-(2-hydroxyethyl)-2,4-dinitrobenzenesulfonamide (1). In a 100 mL round bottom (RB) flux, ethanol amine (0.8 g, 13.2 mmol) was dissolved into dry DCM in a nitrogen atmosphere. The RB was placed in an ice-water bath under constant stirring. Et_3N (2.2 mL,

19.2 mmol) was added to the solution and kept under stirring condition for another 30 min. 2,4-DNSCl (3.4 g, 19.2 mmol) was dissolved into dry DCM and added dropwise to the solution over 10 min and left overnight for stirring. The reaction mixture was then diluted with DCM and washed with water (3 × 50 mL) followed by brine solution (50 mL) and dried over $MgSO_4$. The crude mixture was purified through silica gel column chromatography with 2% MeOH in DCM to obtain pure **1**, yield = 75%. ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm, Figure S1): 9.17 (d, J = 2.7 Hz, 1H), 8.84 (s, 1H), 8.30 (ddd, J = 9.5, 2.7, 0.7 Hz, 1H), 7.01 (d, J = 9.5 Hz, 1H), 4.04 (dd, J = 5.7, 4.9 Hz, 2H), 3.63 (q, J = 5.3 Hz, 2H).

Synthesis of 2-((2,4-dinitrophenyl)sulfonamido)ethyl methacrylate (M) monomer. In a 100 mL RB flask, methacrylic acid (0.15 g, 1.40 mmol) was dissolved into DCM and kept under stirring in an ice-water bath. To this solution, DCC (3.40 g, 1.60 mmol) and DAMP (0.04 g, 0.24 mmol) were added. After 30 min, compound **1** (0.40 g, 1.40 mmol) in DCM was added dropwise to the above solution over 10 min and kept under stirring for 24 h. The mixture was filtered to remove DCU and diluted with 200 mL DCM followed by washing with water (3 × 100 mL) and brine solution (100 mL). The solution was dried over $MgSO_4$ and concentrated under rotatory evaporation. The crude mixture was purified through silica gel column chromatography with 15% ethyl acetate in hexane to obtain pure monomer **M**, yield = 60%. ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm, Figure S2): 9.16 (d, J = 2.7 Hz, 1H), 8.79 (s, 1H), 8.31 (ddd, J = 9.5, 2.6, 0.7 Hz, 1H), 7.01 (d, J = 9.5 Hz, 1H), 6.16 (s, 1H), 5.65 (s, 1H), 4.50-4.47 (m, 2H), 3.76 (q, J = 5.6 Hz, 2H), 1.96 (s, 3H). ^{13}C NMR (300 MHz, $CDCl_3$, δ ppm, Figure S3) δ 167.23, 148.37, 135.62, 130.58, 127.06, 124.44, 113.92, 62.00, 49.29, 42.45, 34.07, 25.41 (d, J = 201.7 Hz), 18.39.

Cell viability assay of BCP1Np-BCP3Np. The cell viability of HeLa Kyoto cell in response to varying polymer concentrations was assessed using the MTT assay,[3] which relies on the conversion of MTT by mitochondrial active succinate dehydrogenase, resulting in the formation of purple-colored formazan crystals. The absorbance at 570 nm directly correlates with the quantity of viable cells. For this experiment, 10000 cells were seeded in a 96-well plate with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Cells were exposed to 6 different polymer concentrations: 0.01, 0.05, 0.1, 0.2, 0.3, and 0.4 mg/mL for the three polymeric nanoparticles **BPC1Np**, **BPC2Np**, and **BPC3Np**. Additionally, one well served as a control without polymer. The cells were incubated at 37 °C with 5% CO_2 for 24 h. Next, the cells in each well were treated with MTT (2 mg/mL) for 3 h at 37 °C with 5% CO_2. Following this, 100 μL of DMSO was introduced into each well, and the absorbance at 570 nm was subsequently determined using a Spectra Max iD5 spectrometer (Molecular Devices). The experiment was repeated thrice, and the resulting data were analyzed. Error bars, indicative of standard deviation, were incorporated into the evaluation of cell viability in polymer-treated conditions, with cells grown in DMEM complete media without polymer serving as the control.

Evaluation of antibacterial activity

Determination of MIC_{50} Value. The MIC_{50} was assessed against *B. subtilis* (Gram-positive) and *E. coli* (Gram-negative) bacteria through a broth micro dilution assay, employing the logarithmic growth phase (OD_{600} = 0.6-1.0) of both bacteria.[4] Bacterial cultures of *B. subtilis* and *E. coli* were propagated in liquid nutrient LB medium, prepared by dissolving 15 g of LB in 1000 mL of Milli-Q water. The assays were conducted with an initial bacterial concentration of 5 × 10^5 CFU/mL. Different concentrations of bacteria suspensions were prepared by adding 12.5 to 400 μL of **BCP3Np** solutions in LB broth, maintaining a total

volume of 1 mL, and incubated at 37 °C for 12 h. Subsequently, OD of the samples were collected at 600 nm employing a Cary Varian 50 scan UV-vis optical spectrometer. Growth OD% was determined by the following equation (1):

Growth OD (%) = $[(OD_{\textbf{BCP3Np}\ \text{treated}} - OD_{\text{positive control}})/(OD_{\text{blank}} - OD_{\text{positive control}})] \times 100\%$ (1)

where, OD_{blank} corresponds to negative control (without **BCP3Np**) and $OD_{\text{positive control}}$ corresponds to 0.1% Triton X used as positive control. Each concentration was examined three times, and the entire experiment was repeated at least three times. The bacterial growth inhibition graph was plotted with average triplicate OD values against polymer concentration. The MIC_{50} value of the polymers was determined using GraphPad Prism 7.04 employing a four-parameter fitting model.[5]

Agar plate imaging. Typically, 90 mm sterile plastic plates were employed to prepare the gel bed consisting of sterilized 1.5% LB and 2% agar.[6] The nutrient broth and agar medium were sterilized at 121 °C for 30 min. *B. subtilis* and *E. coli* were cultured at 37 °C for 18 h in a liquid nutrient LB medium with different concentrations of **BPC3Np** such as 0.01, 0.05, 0.08, 0.1, and 0.3 mg/mL. Next, 30 mL of bacterial suspension and polymer treated bacterial solutions were spread on the plates and incubated at 37 °C for 24 h, and the photographic images were collected.

Colony forming unit (CFU) assay. Bacterial cultures of *B. subtilis* and *E. coli* were propagated in liquid nutrient LB medium, prepared by dissolving 15 g of LB in 1 L of Milli-Q water. The colony forming unit was assessed against *B. subtilis* (Gram-positive) and *E. coli* (Gram-negative) bacteria through a broth micro dilution assay, employing the logarithmic growth phase (OD_{600} = 0.6-1.0) of both bacteria.[7] The assays were conducted with an initial bacterial concentration of 4.5×10^5 CFU/mL. A fixed volume of different concentration solutions (0.05, 0.1, 0.2 and 0.4 mg/mL) of **BCP3Np** were mixed with bacterial suspension and incubated for 18 h at 37 °C (4.5×10^5 CFU/mL concentration was used as control for both

the bacteria). Next, the **BCP3Np** treated and nontreated bacteria solutions were spread on the agar plate, incubated for another 18 h at 37 °C and the photographic images of plates were collected.

ROS generation study in B. subtilis. Bacterial ROS generation in the presence of polymer was assessed using DCFDA, which shows fluorescence maxima at 535 nm upon excitation at 485 nm in its activated form.[8] Initially, 1 mL of an overnight-cultured *B. subtilis* bacterial suspension was centrifuged at 6000 rpm for 5 min, resulting in the formation of a pellet at the bottom of the tube. The supernatant was removed, and the sediment was re-suspended in HBSS. Subsequently, this new bacterial suspension was treated with varying concentrations of the **BPC3Np**, ranging from 10 to 300 μg/mL, followed by a 6 h incubation period. A 2 M stock solution of DCFDA was prepared in DMSO and 10 μL of this stock solution was added to each micro-centrifuge tube. The samples were incubated for 10 min and fluorescence readings were acquired using a Fluoromax-3 instrument. This experimental setup is aimed to observe and quantify the impact of various concentrations of the **BPC3Np** on ROS generation in *B. subtilis*, as indicated by changes in fluorescence emission at 535 nm.

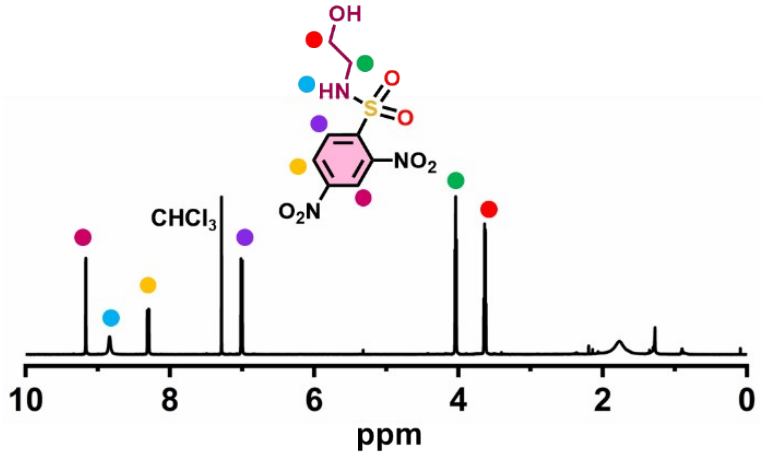

Fig. S1 ^{1}H NMR spectrum of **1** in $CDCl_3$.

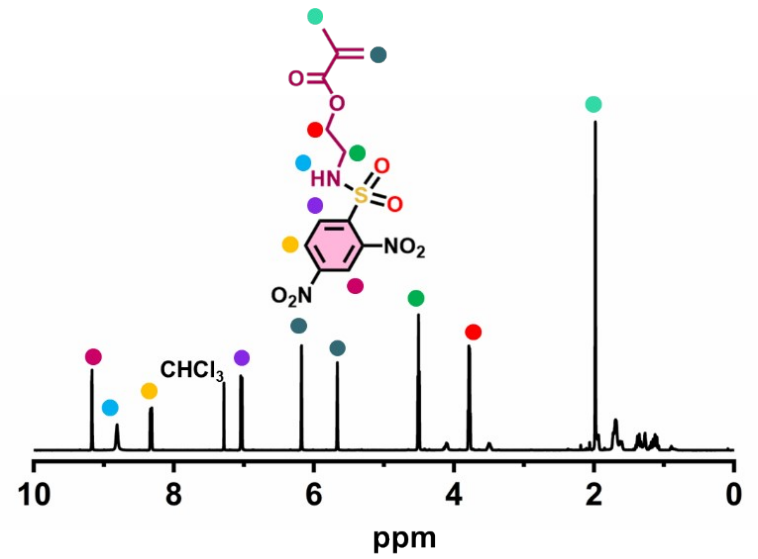

Fig. S2 ^{1}H NMR spectrum of **M** in $CDCl_3$.

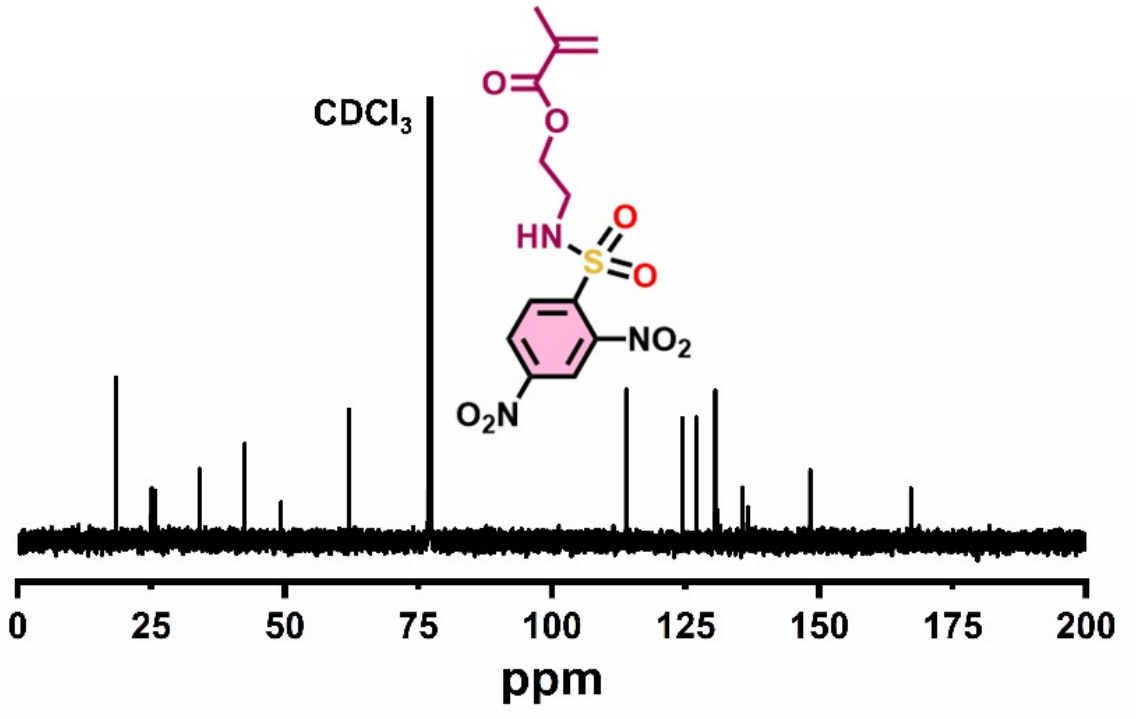

Fig. S3 ^{13}C NMR spectrum of **M** in $CDCl_3$.

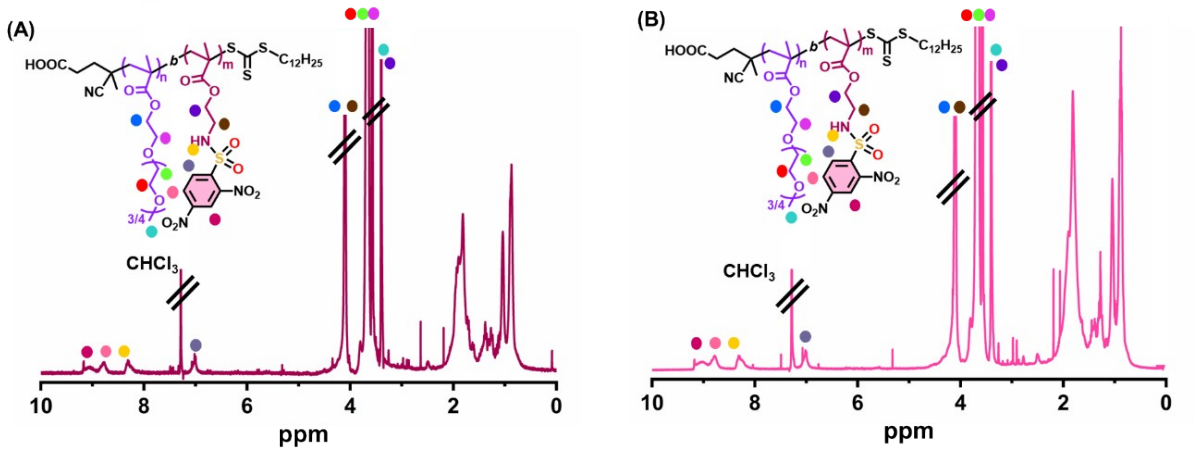

Fig. S4 (A) ^{1}H NMR spectra (A) **BCP1** and (B) **BCP2** recorded in $CDCl_3$.

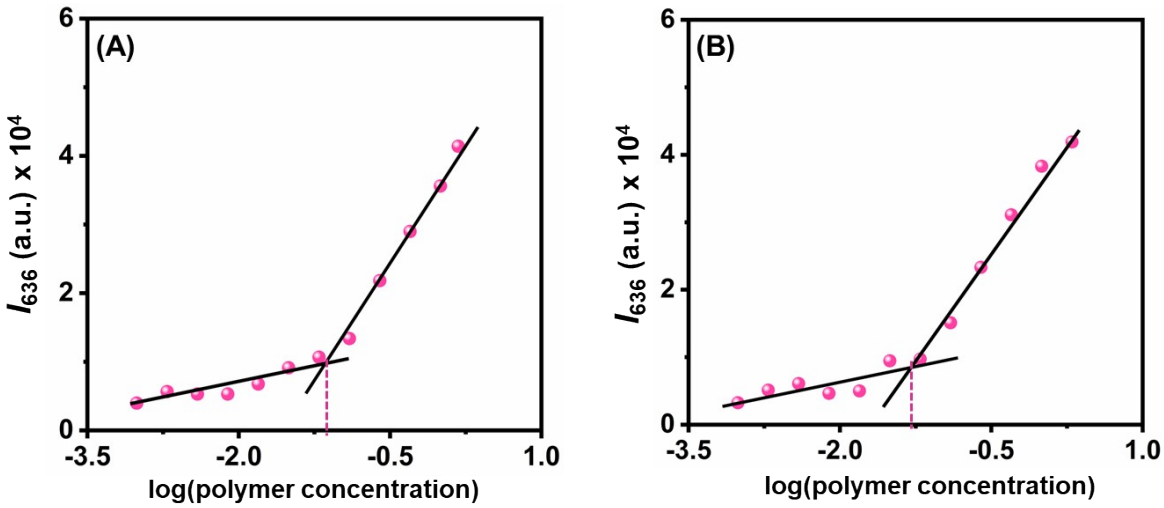

Fig. S5 (A) CAC determination of (A) **BCP1** and (B) **BCP2** in aqueous medium.

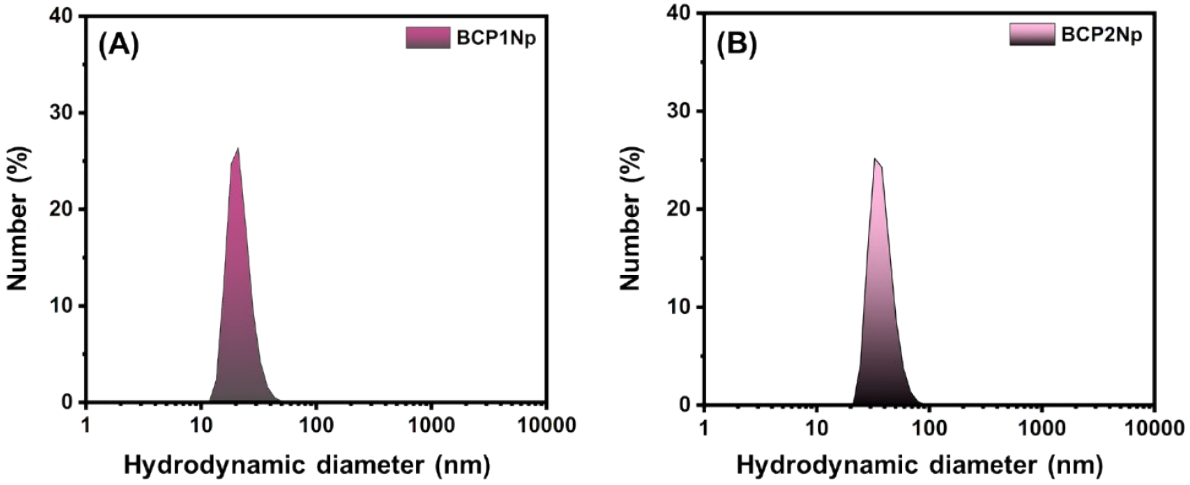

Fig. S6 DLS size distribution profile of (A) **BCP1Np** and (B) **BCP2Np** in aqueous medium (concentration = 0.5 mg/mL).

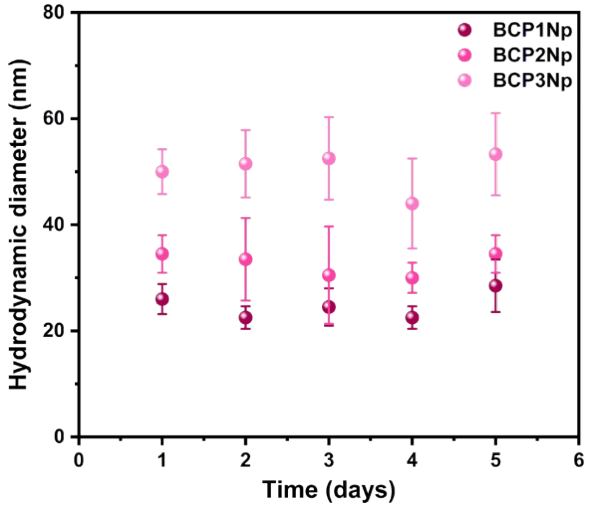

Fig. S7 Stability study of polymeric nanoparticles in water. The polymer solutions (0.5 mg/mL) were kept at 25 °C and the hydrodynamic diameters were measured at regular time intervals.

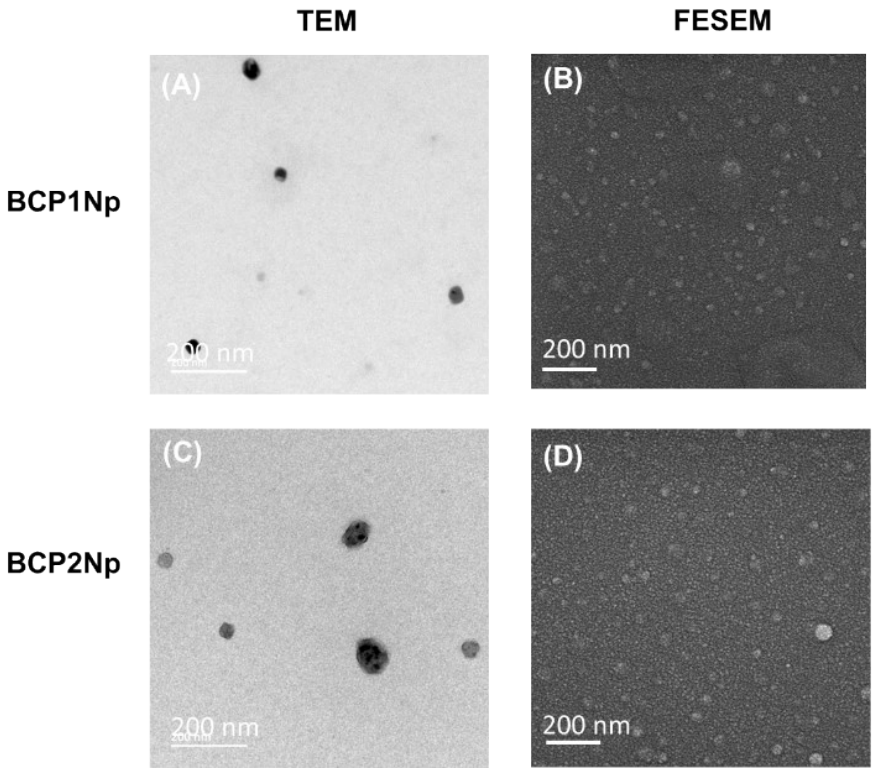

Fig. S8 TEM and FESEM images of (A, B) **BCP1Np** and (C, D) **BCP2Np**.

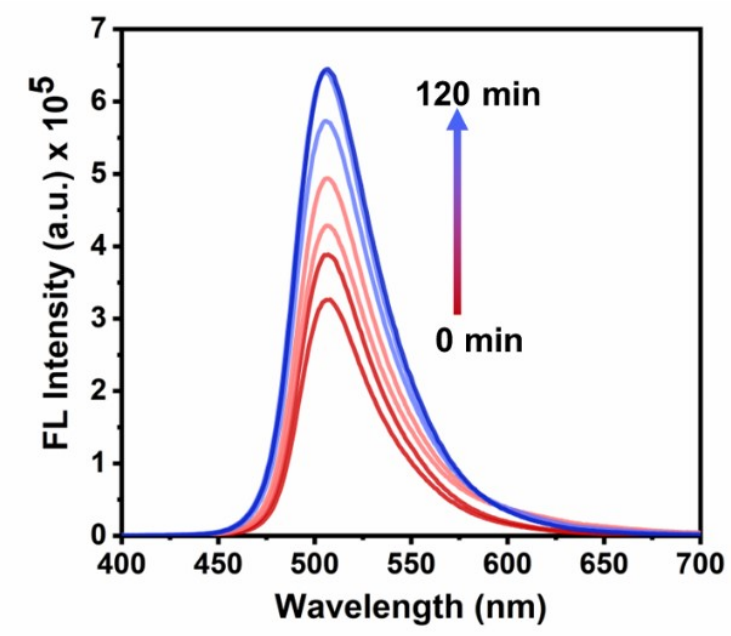

Fig. S9 Release of SO_2 from compound **1** in the presence of GSH in DMSO/PBS buffer (1:1, v/v) using DEACA as a fluorescent probe.

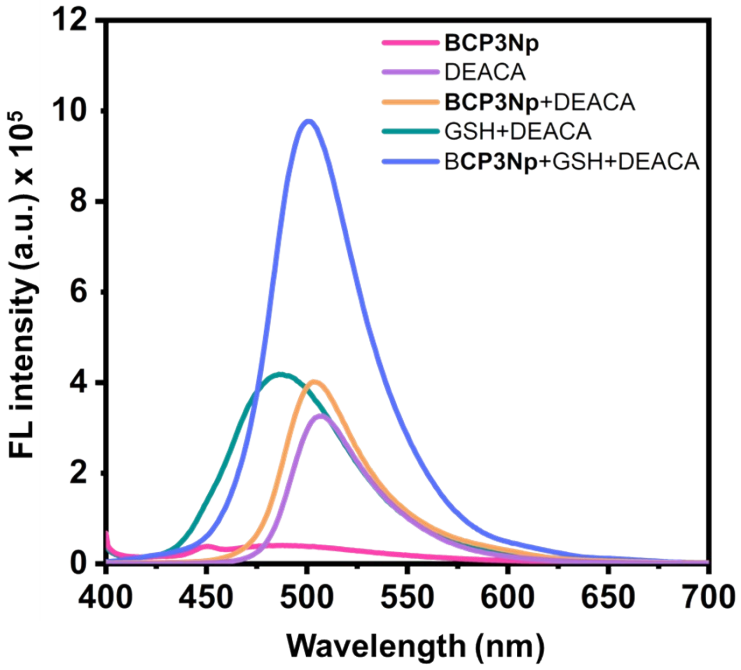

Fig. S10 SO_2 release study from **BCP3Np** with and without GSH in PBS buffer using DEACA as a fluorescent probe.

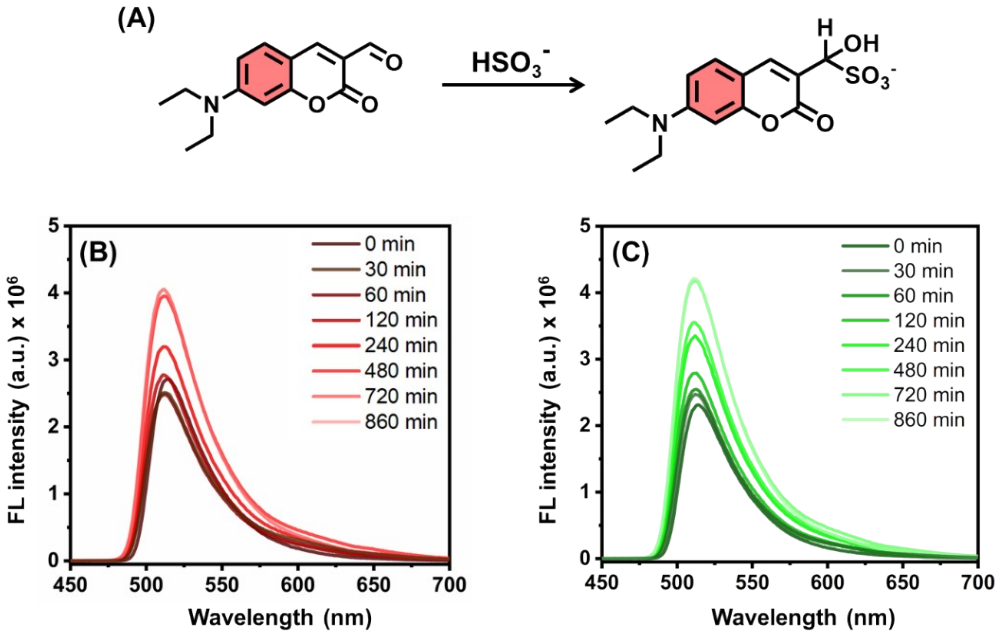

Fig. S11 (A) Representation of detection of SO_2 with the DEACA probe. Time kinetics for detection of SO_2 derivative HSO_3^- in PBS solution of (B) **BCP1Np** and (C) **BCP2Np** in the presence of GSH (10 mM) using DEACA as a fluorescent probe.

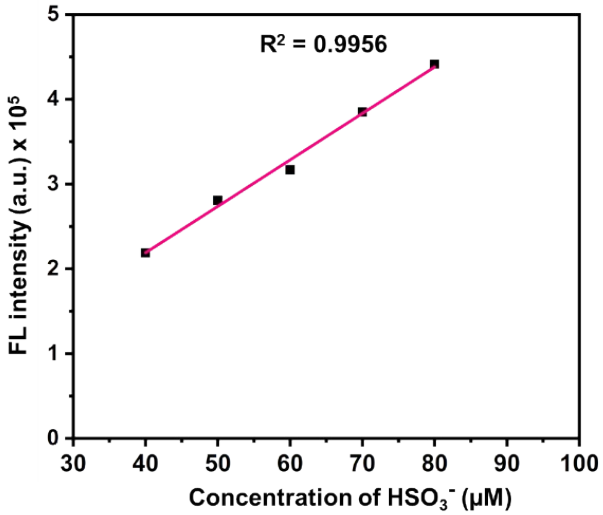

Fig. S12 Generation of the standard curve by plotting fluorescence intensity as a function of concentrations of HSO_3^-.

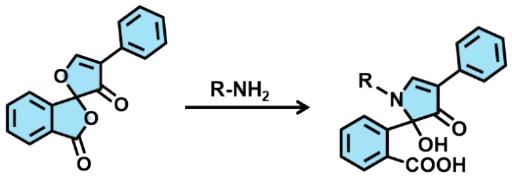

Fig. S13 Representation of detection of free primary amine by fluorescamine.

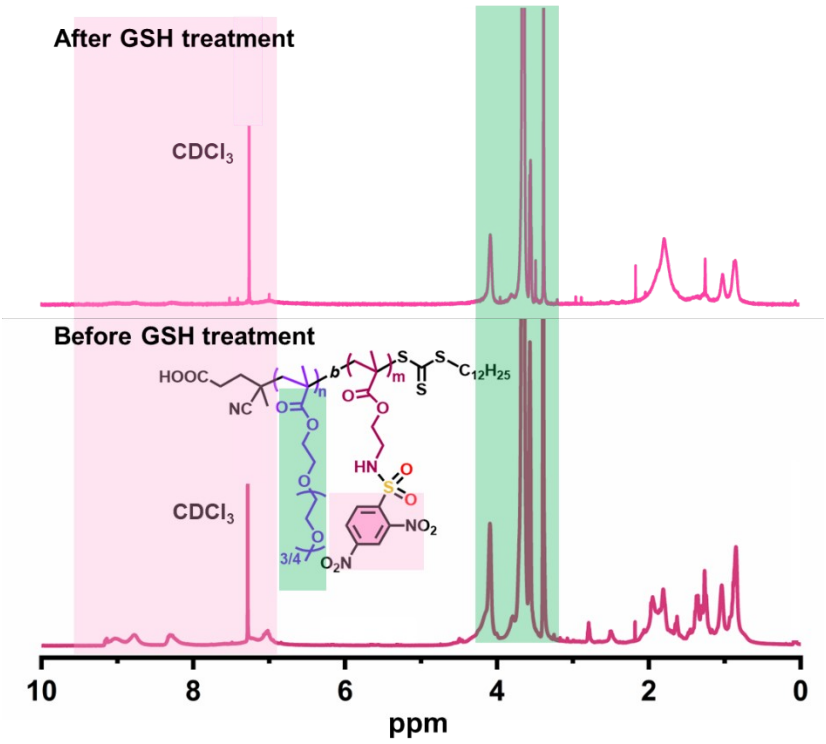

Fig. S14 ^{1}H NMR spectra of **BCP3Np**, before (bottom) and after (top) reaction with GSH recorded in $CDCl_3$.

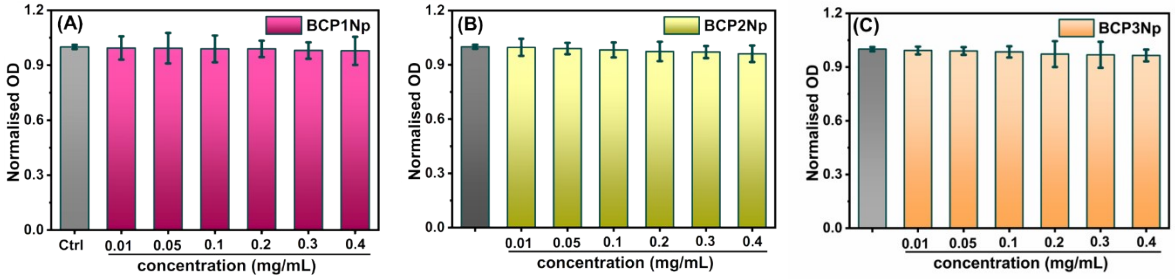

Fig. S15 Cytotoxicity assay at different concentrations of (A) **BCP1Np**, (B) **BCP2Np**, and (C) **BCP3Np** on HeLa Kyoto cells.

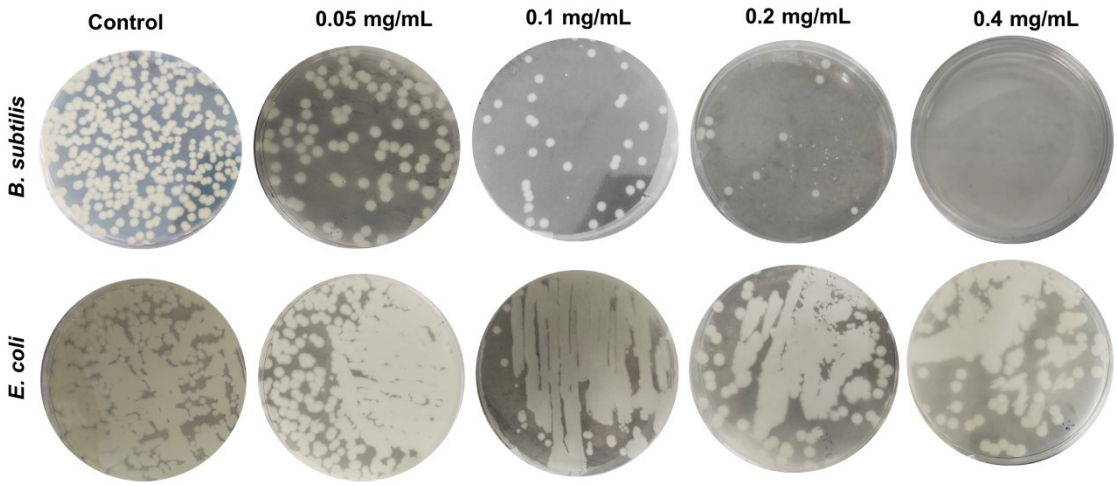

Fig. S16 The bactericidal activity of **BCP3Np** against *B. subtilis* and *E. coli*.

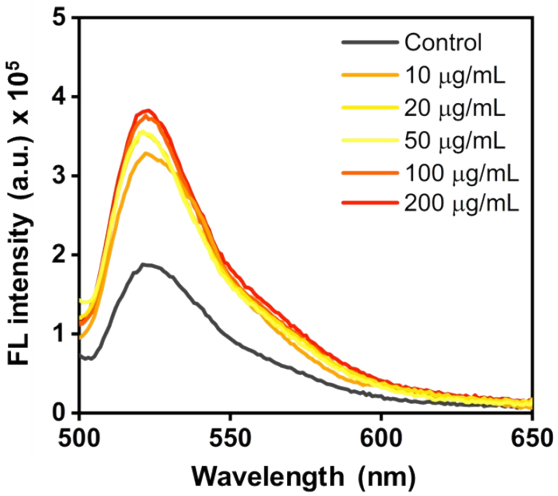

Fig. S17 The fluorescence spectra of 2',7'-dichlorofluorescein (DCF) probing the intracellular ROS generation in *B. subtilis* in the presence of different concentrations of **BCP3Np**.

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