Electronic Supplementary Material (ESI) for Biomaterials Science.


*Supporting Information*

# Supramolecular Pyrrole Radical Cations for Bacterial Theranostics

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## Experimental Section

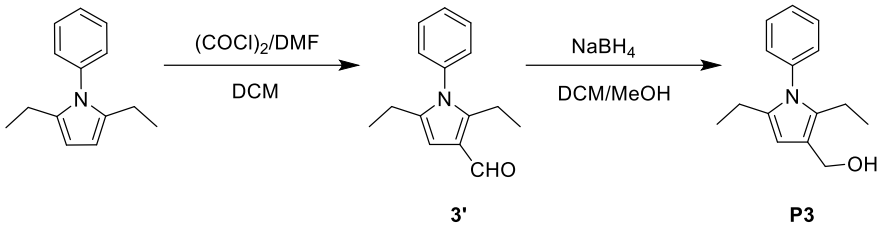
### Materials and chemicals

Pyrrole compounds P1, P2, P4, and P5 are prepared according to our previous reports.[1] Aniline, 4-nitroaniline, *p*-anisidine, acetonylacetone, oxalyl chloride, piperidine, sodium bicarbonate, colistin sulfate, vancomycin and tetrahydrofuran (THF) were purchased from Macklin (Shanghai, China) and used as received without further purification. Sodium borohydride ($NaBH_4$), iodine, sodium acetate trihydrate, acetic acid, dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF) were purchased from Aladdin and used as received without further purification. Methanol (MeOH) was purchased from Tianjin Chemical Reagent Factory. Cucurbit[7]uril (CB[7]) was purchased from ShangHai SuperLan Chemical Tech Centre. Ultrapure water was supplied by a Milli-Q Plus System (Millipore Corporation, United States). Dulbecco's Modified Essential Medium (DMEM), fetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from Gibco (Life Technologies).

### Equipment and methods

The UV–vis absorption spectra and photoluminescence spectra were measured on a microplate reader (Tecan Infinite M200 PRO). The $^{1}H$ and $^{13}C$ NMR spectra were measured on a Bruker AV 400 NMR spectrometer. The high-resolution mass spectra (HRMS) were recorded on an Agilent 7250 operated in the EI mode. Confocal laser scanning microscopy (CLSM) images were obtained on a confocal microscope (Zeiss Laser Scanning Confocal Microscope, LSM880, Germany). Flow cytometry was measured on a BD FACScelesta system. Zeta potential measurements were obtained on a Malvern Zetasizer. The electron paramagnetic resonance (EPR) spectra were measured on a Bruker E500-10/12 electron paramagnetic resonance CW. The absolute fluorescence quantum yield was measured on an FLS1000 fluorescence spectrophotometer (Edinburgh Instruments Ltd, U.K). The fluorescence lifetime was measured on a Hamamatsu Compact Fluorescence Lifetime Spectrometer C11367.

**Synthesis of pyrroles**




**Scheme S1.** Synthetic route of compounds P3

**Synthesis of compound 3′**

Oxaloyl chloride (512 μL, 6.0 mmol) was added slowly into a flask loaded with *N*,*N*-dimethylformamide (DMF) (464 μL, 6.0 mmol) and stirred for 10 min in an ice bath. Compound 2,5-diethyl-1-phenyl-1*H*-pyrrole (299 mg, 1.5 mmol) dissolved in DCM (15 mL) was dropped and reacted at room temperature for 20 min. An aqueous solution (10 mL) of sodium acetate (1.23 g, 15.0 mmol) was added and reacted at room temperature for 3 h. The reaction mixture was extracted and further separated by column chromatography (silica, petroleum ether/ethyl acetate = 3/1) to afford compound 3′ (245 mg, 72% yield). $^1$H NMR (400 MHz, $d_6$-DMSO): $\delta$ 9.83 (s, 1H), 7.61-7.56 (m, 3H), 7.39-7.37 (m, 2H), 6.30 (s, 1H), 2.66 (q, $J$ = 7.6 Hz, 2H), 2.21 (q, $J$ = 7.6 Hz, 2H), 1.02 (t, $J$ = 7.6 Hz, 3H), 0.93 (t, $J$ = 7.6 Hz, 3H). EI-HRMS: m/z $[M + H]^+$ calcd. for $C_{15}H_{17}NO^+$, 227.1310; found, 227.1306.

**Synthesis of compound P3**

Compound 4′ (159 mg, 0.7 mmol) was first dissolved in methanol (5 mL), and $NaBH_4$ (79 mg, 2.1 mmol) was then added. The mixture was stirred in an ice bath for 30 min in argon. After completion of the reaction, the reaction mixture was extracted and further dried under reduced pressure to afford compound P3 (142 mg, 89% yield). $^1$H NMR (400 MHz, MeOD): $\delta$ 7.52-7.50 (m, 3H), 7.24-7.22 (m, 2H), 5.95 (s, 1H), 4.49 (s, 2H), 2.46 (q, $J$ = 7.6 Hz, 2H), 2.30 (q, $J$ = 7.6 Hz, 2H), 1.04 (t, $J$ = 7.2 Hz, 3H), 0.89 (t, $J$ = 7.6 Hz, 3H); $^{13}$C NMR (100 MHz, MeOD):

$\delta$ 139.0, 134.2, 132.6, 128.8, 128.6, 127.7, 117.9, 104.9, 56.5, 19.6, 17.3, 14.5, 12.5. EI-HRMS: m/z $[M + H]^+$ calcd. for $C_{15}H_{19}NO^+$, 229.1467; found, 229.1463.

**Preparation of bacterial suspensions**

A single colony of *S. aureus* or *E. coli* on a solid nutrient broth (NB) agar plate was transferred to 5 mL of liquid LB culture medium and grown at 37 °C for 6 ~ 8 h with a shaking speed of 220 rpm. *S. aureus* or *E. coli* was harvested by centrifugation for 3 min at 8000 rpm and washed with PBS three times. After removal of the supernatant, *S. aureus* or *E. coli* was resuspended in PBS and diluted to an optical density ($OD_{600}$) of 1.0 at 600 nm.

**SEM and TEM characterization of bacteria morphology**

Two hundred microliters of *S. aureus* ($OD_{600}$ = 1.0) stained with $P^{\bullet+}$-CB[7] ([P1] = 100 μM, [CB[7]] = 33.3 μM) was fixed with 4% paraformaldehyde solution for 12 h at 4 ℃. Then, the samples were harvested by centrifugation for 3 min at 8000 rpm and washed with PBS three times. The samples were dehydrated by different contents of ethanol (30%, 50%, 60%, 70%, 80%, 90%, 95%, and 100%) for 30 min. For SEM characterization, two microliters of the sample was dropped onto a clean silicon wafer. After drying at room temperature, the samples were coated with platinum and further observed under scanning electron microscopy (SEM, Zeiss Merlin). For TEM characterization, two microliters of the sample was dropped onto a clean TEM copper grid and dried at room temperature. The samples were further observed under transmission electron microscopy (TEM, Talos L120c, Thermo Fisher).

**Cell culture**

RAW264.7 cells were provided by American Type Culture Collection (ATCC) and cultured in DMEM (Dulbecco's Modified Essential Medium) with 1% penicillin–streptomycin and 10% FBS (fetal bovine serum) at 37 °C in a humidified incubator with 5% $CO_2$ at 37 ℃.

L929 cells were purchased from Procell Life Science & Technology Co., Ltd. and cultured in DMEM (Dulbecco's Modified Essential Medium) with 1% penicillin–streptomycin and 10% FBS (fetal bovine serum) at 37 °C in a humidified incubator with 5% $CO_2$ at 37 ℃.

**Cell viability assay**

The cell viability was assessed by MTT assay. The L929 cells were seeded into 96-well plates ($1 \times 10^4$ cells/well) for 24 h. Subsequently, the cells were rinsed with PBS and further treated with various concentrations of P-CB[7]. The cells were further incubated for 24 h. Finally, after washing with PBS buffer for twice, MTT solution (0.5 mg $mL^{-1}$, 100 μL) was added. After further incubation at 37 °C for 4 h, the MTT solution was removed, and the cells were washed twice with PBS buffer. DMSO (100 μL) was then added into each well to dissolve all the precipitates for 10 min. The OD values at 570 nm were obtained by a microplate reader and cell viability was then evaluated.

**Cytoplasmic membrane depolarization assay**

*S. aureus* was washed with HEPES buffer (5 mM HEPES, 20 mM glucose, pH=7.4) and suspended in HEPES buffer at $1\times10^7$ CFU $mL^{-1}$. The bacterial suspension was incubated with 0.8 μM DiSC3(5) followed by adding 1 mM KCl to equilibrate the cytoplasmic and external $K^+$. An aliquot of 90 μL of the cell suspension was placed in a 96 well plate. Changes in fluorescence were recorded on microplate reader ($\lambda_{ex}$ = 622 nm, $\lambda_{em}$ = 673 nm). When the fluorescence intensity was almost steady, P-CB[7] was added into the system (Time = 0 s) with a final concentration of 300 and 600 μM. The fluorescence intensities were recorded continuously. 0.1% Triton X-100 was used as a positive control.

**In situ monitoring of phagocytosis of bacteria**

One day before the phagocytosis experiment, RAW264.7 cells were plated in a glass bottom confocal Petri dish at a density of $1 \times 10^5$ cells/well, and the cells were incubated for 24 h at 37 °C with 5% $CO_2$. Then, the medium was replaced with 1 mL medium containing $P1^{\bullet+}$-CB[7] ([P1] = 100 μM, [CB[7]] = 33.3 μM) stained with *S. aureus* at a quantity ratio of 8/1

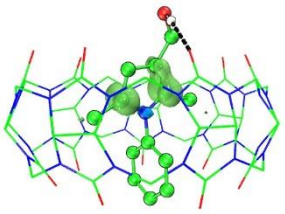
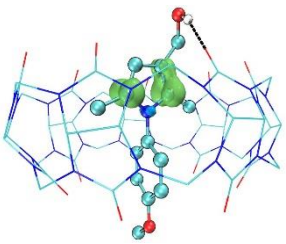
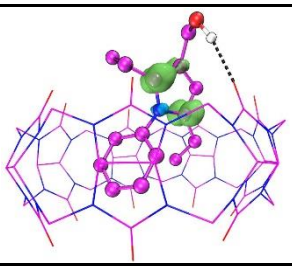
(bacteria/macrophages). After 1 h, the medium was removed, and the samples were washed with PBS 3 times to remove bacteria outside of macrophages. Then, the phagocytosis of bacteria by macrophage RAW264.7 cells was monitored in situ under a confocal microscope. $\lambda_{ex}$ = 633 nm, $\lambda_{em}$ = 650-750 nm.

**Table S1.** The photophysical properties of compound $P^{\bullet+}$-CB[7].

| Compound | $\lambda_{ex}$ [nm][c)] | $\lambda_{em}$ [nm][d)] | $\Phi_F$ [%][e)] | $\tau$ (ns)[f)] | $k_r$ [$10^7$ $s^{-1}$][g)] | $k_{nr}$ [$10^8$ $s^{-1}$][h)] |
|---|---|---|---|---|---|---|
| $P1^{\bullet+}$-CB[7][a)] | 600 | 675 | 1.67 | 1.31 | 1.27 | 7.51 |
| $P2^{\bullet+}$-CB[7][b)] | 600 | 670 | 1.30 | 0.79 | 1.65 | 12.49 |

a) $P1^{\bullet+}$-CB[7] was prepared in ultrapure water with a molar ratio of P1:CB[7] = 3:1, [P1] = 1 mM, [CB[7]] = 333 μM. b) $P2^{\bullet+}$-CB[7] in ultrapure water with a molar ratio of P2:CB[7] = 3:1 and [P2] = 1 mM, [CB[7]] = 333 μM. c) Maximum absorption wavelength. d) Maximum emission wavelength. e) Absolute quantum yield. f) Average fluorescence lifetime measured under ambient conditions. g) Radiative relaxation rate $k_r = \Phi/\tau$. h) Nonradiative relaxation rate $k_{nr} = (1-\Phi)/\tau$.

**Table S2.** Theoretical calculation of $P^{\bullet+}$-CB[7].

| Complex | $P1^{\bullet+}$-CB7 | $P2^{\bullet+}$-CB7 | $P4^{\bullet+}$-CB7 |
|---|---|---|---|
| Optimized structure based on DFT calculation |  |  |  |
| $\lambda_{em,\,max}$ (nm) | 717.78 | 713.40 | 695.44 |
| f | 0.0113 | 0.0122 | 0.0123 |

The density functional theory (DFT) method was conducted for the theoretical evaluation. The complex was first optimized at B3LYP/SV(P) with D4 correction and the CPCM solvation model (solvent = water) by ORCA (v5.0.3) software.[2] A successive frequency calculation was then performed by the same method to verify the optimized structure referring to a minimum point of the potential surface. The optimized structure was subjected to time-dependent DFT (TD-DFT) calculations at ωB97XD/def2-SVP with the SCRF solvation model (solvent = water) by Gaussian (v16. B01) software.[3] The first three excitation states were taken into consideration.

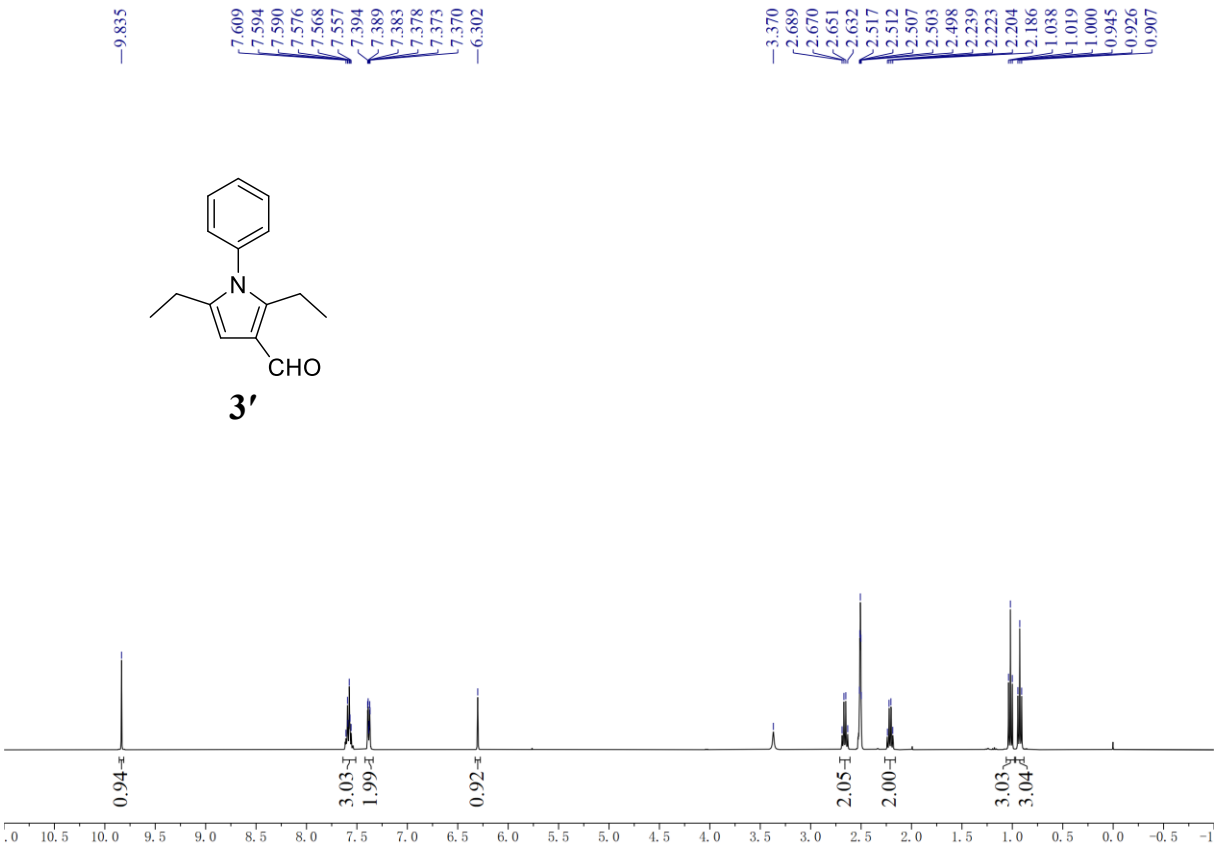



**Figure S1.** $^{1}$H NMR spectrum of compound 3′ in $d_6$-DMSO.

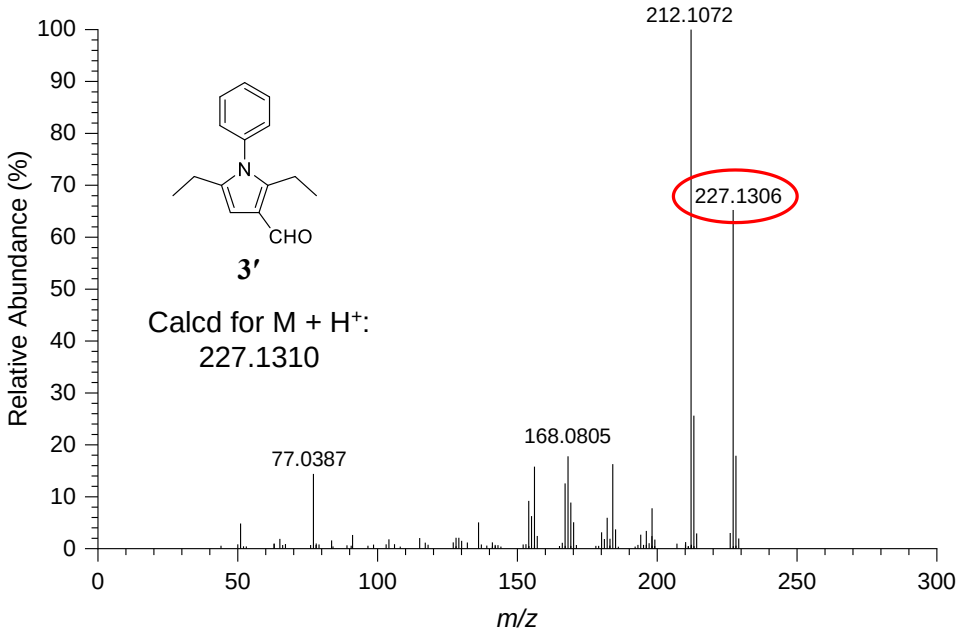



**Figure S2.** HRMS spectrum of compound 3′.

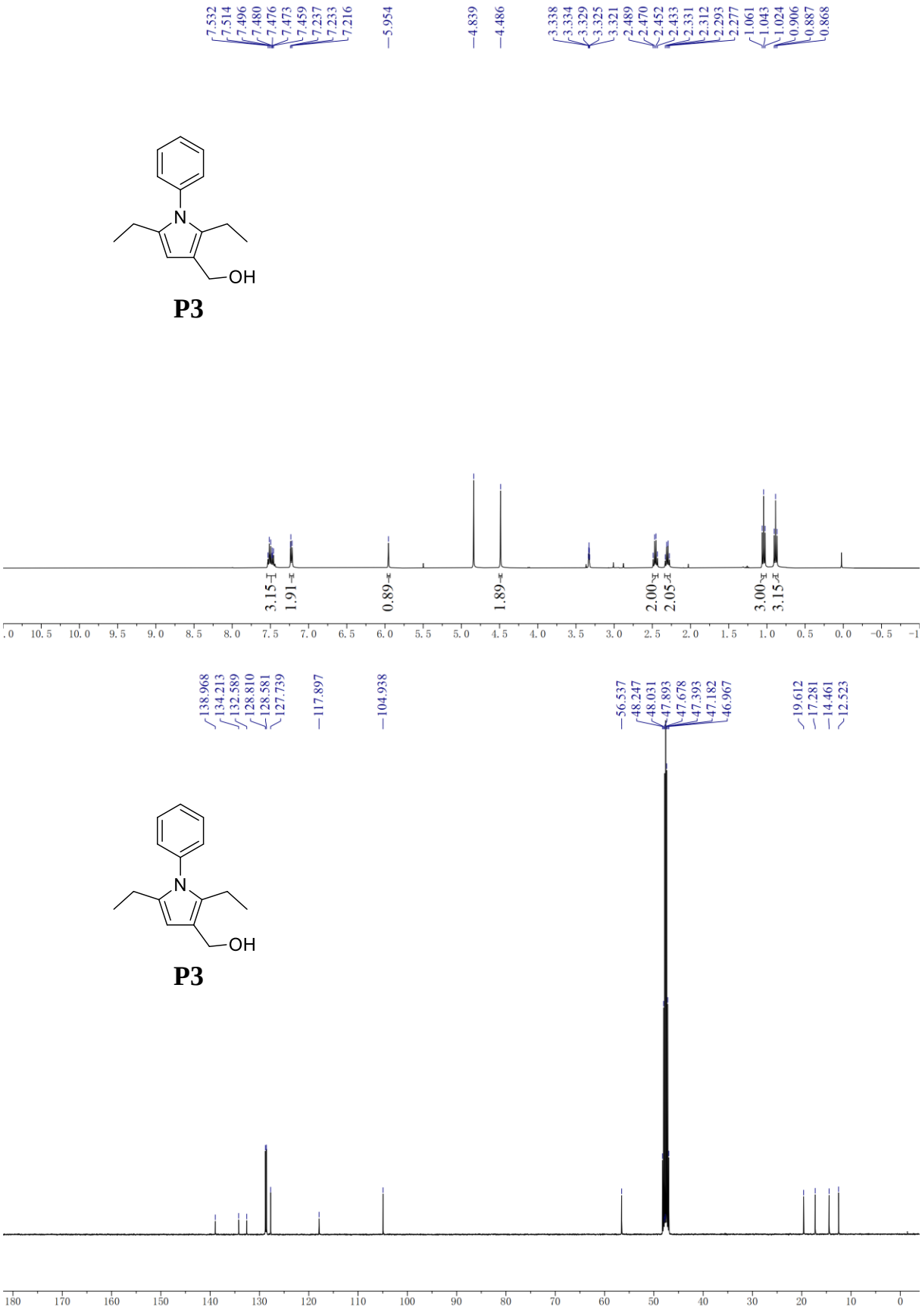



**Figure S3.** $^{1}$H NMR and $^{13}$C NMR spectrum of compound P3 in MeOD.

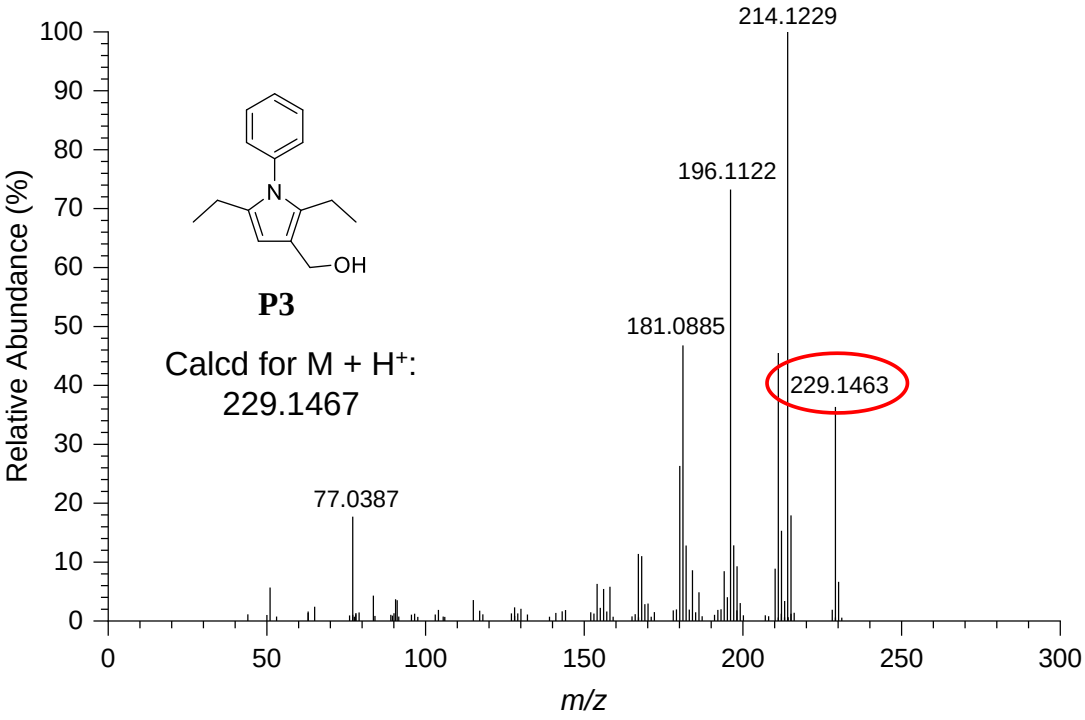



**Figure S4.** HRMS spectrum of compound P3.

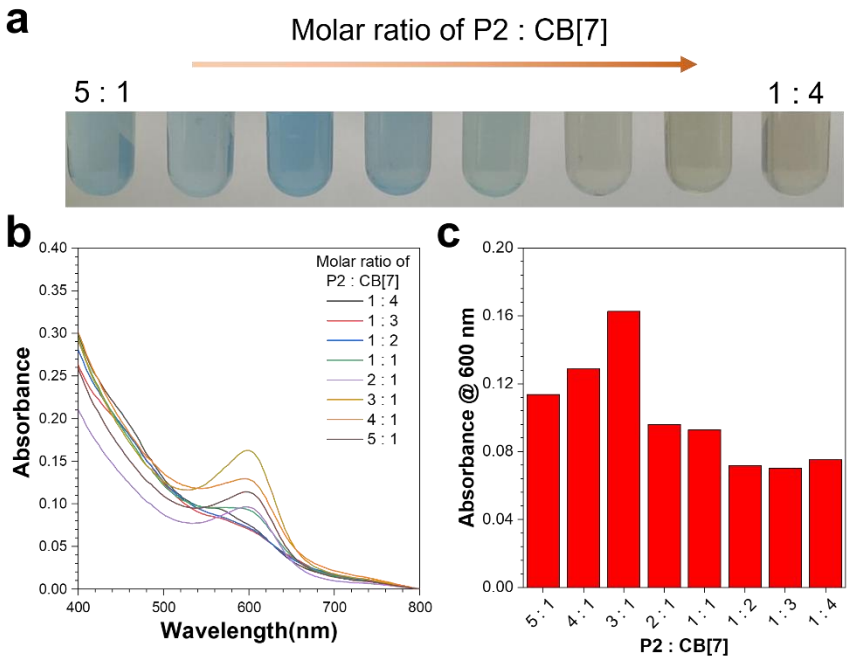



**Figure S5.** Optimization of preparation of pyrrole radical cations by mixing of pyrrole P2 and CB[7] in water at different molar ratios ([P2] = 2.0 mM): a) Photographs under daylight; b) UV–vis absorption spectra; c) The absorbance at 600 nm versus different molar ratios of P2: CB[7].

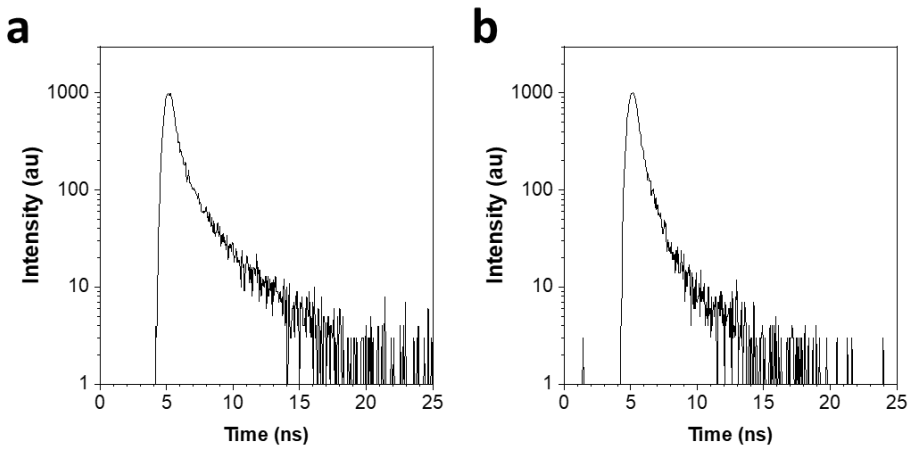



**Figure S6.** Fluorescence lifetime spectra of a) $P1^{\bullet+}$-CB[7] and b) $P2^{\bullet+}$-CB[7] in water.

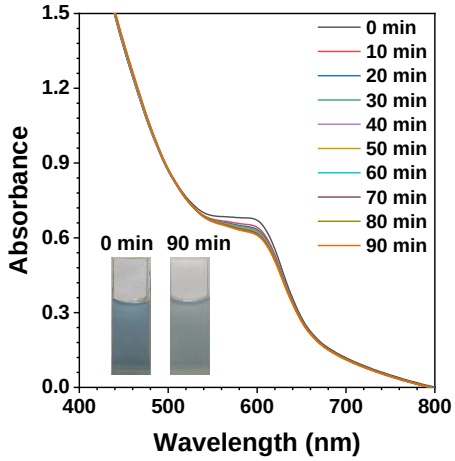



**Figure S7.** Time-dependent UV–vis absorption spectra of $P1^{\bullet+}$-CB[7] in water; [P1] = 3.0 mM, [CB[7]] = 1.0 mM.

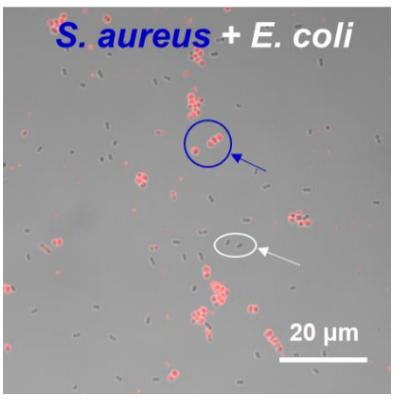



**Figure S8.** CLSM image of mixed *S.aureus* and *E. coli* treated with $P1^{\bullet+}$-CB[7] ([P1] = 100 μM, [CB[7]] = 33.3 μM) for 120 min. Sampled *S.aureus* and *E. coli* is denoted by a blue and white circle, respectively. $\lambda_{ex}$ = 633 nm, $\lambda_{em}$ = 650-750 nm.

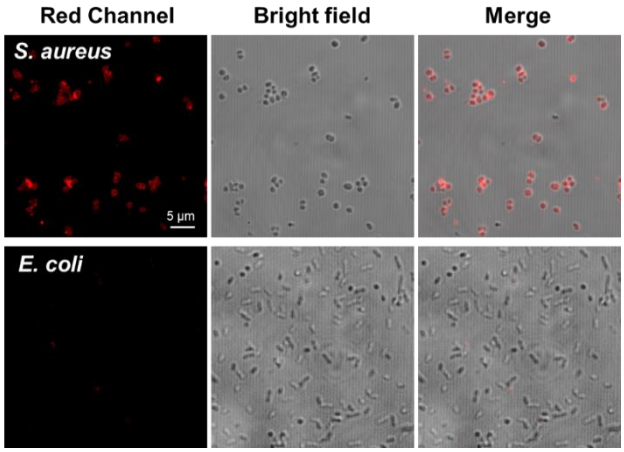



**Figure S9.** CLSM image of *S.aureus* and *E. coli* incubated with P2$^{\bullet+}$-CB[7] for 2 h. For P2$^{\bullet+}$-CB[7], [P2] = 100 μM, [CB[7]] = 33.3 μM, $\lambda_{ex}$ = 633 nm, $\lambda_{em}$ = 650-750 nm.

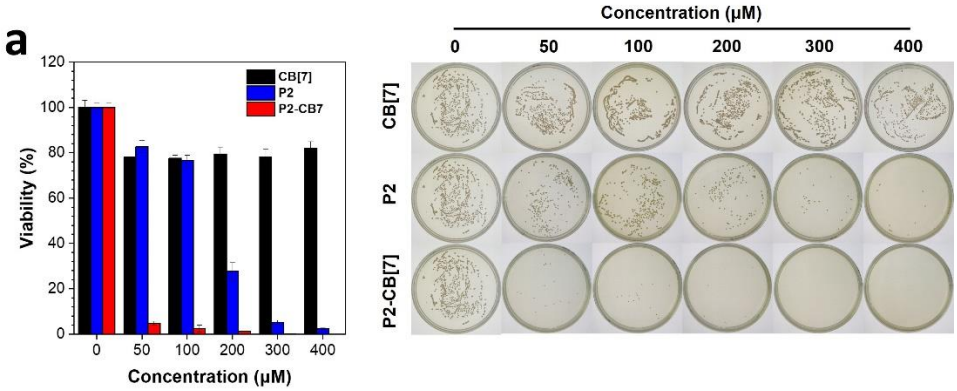



**Figure S10.** a) Bacterial viability and b) photographs of agar plate of *S. aureus* treated with different concentrations of CB[7], P2, and P2-CB[7].

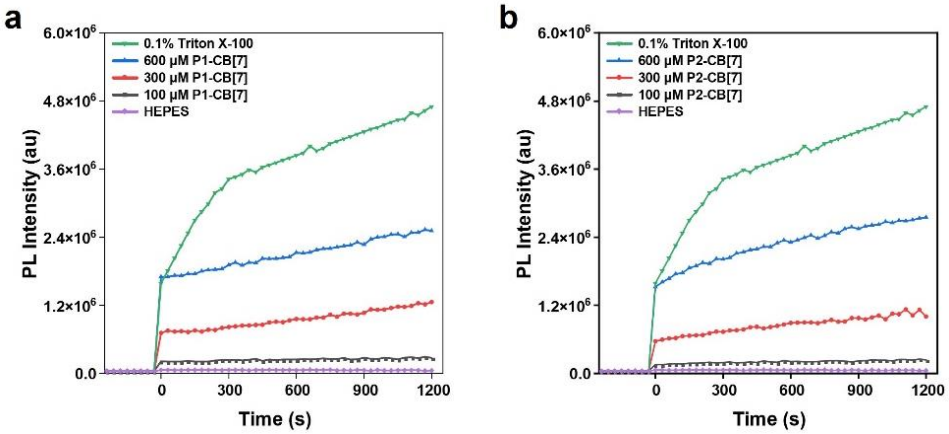



**Figure S11.** Cytoplasmic membrane depolarization of *S. aureus* following treatment with P1-CB[7] and P2-CB[7] at different concentrations.

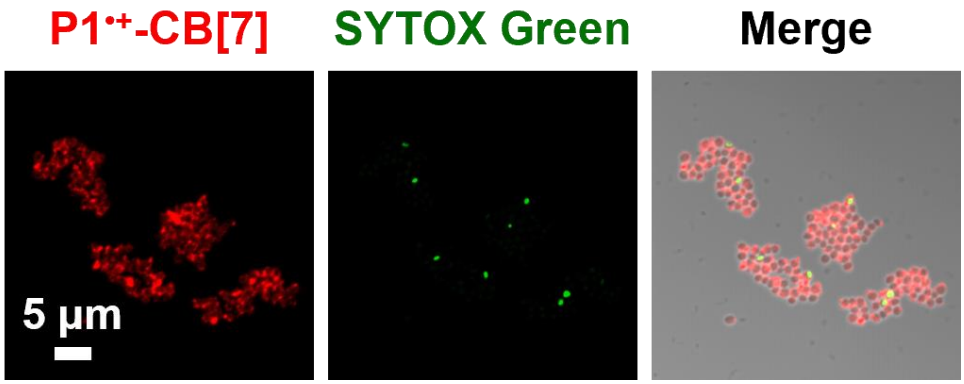



**Figure S12.** CLSM images of *S. aureus* treated with P1$^{\bullet+}$-CB[7] (100 μM) and SYTOX Green (5 μM). For P1$^{\bullet+}$-CB[7], $\lambda_{ex}$ = 633 nm; $\lambda_{em}$ = 650-750 nm; for SYTOX Green, $\lambda_{ex}$ = 488 nm; $\lambda_{em}$ = 490-550 nm.

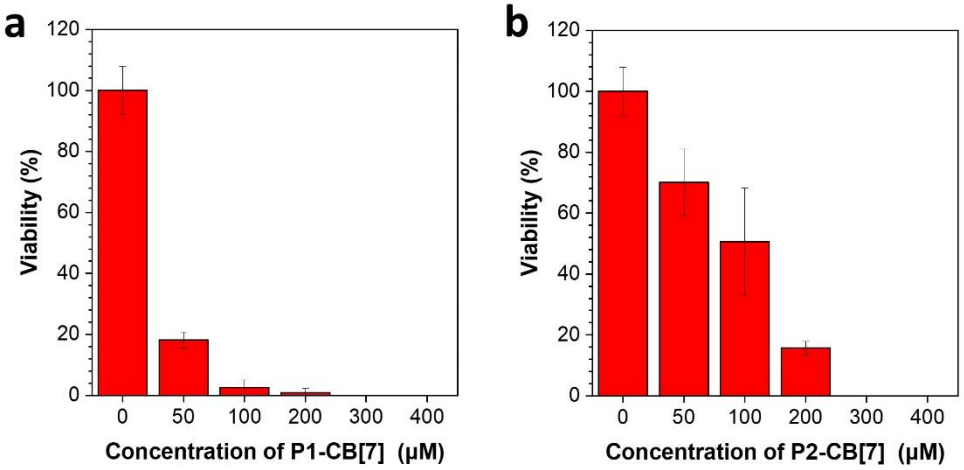



**Figure S13.** Bacterial viability of methicillin-resistant *S. aureus* (MRSA) treated with a) P1-CB[7] and b) P2-CB[7].

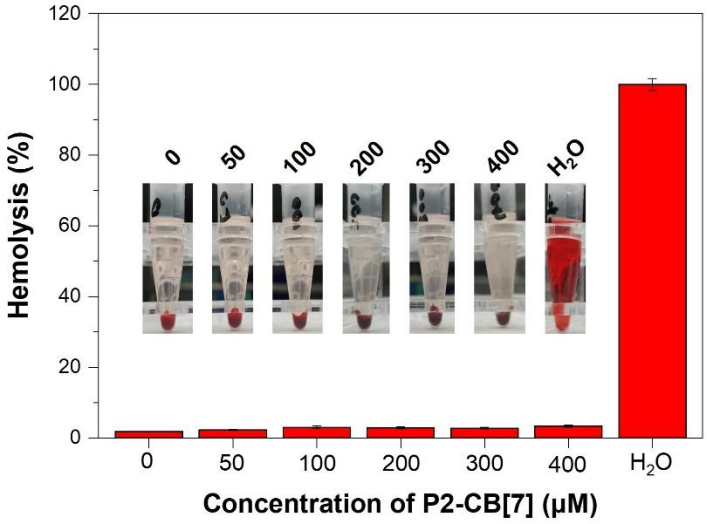



**Figure S14.** Hemolysis (%) was determined by treatment of rabbit red blood cells with P2-CB[7] in PBS, and water treatment was used as a positive control. Inset shows the photographs of the corresponding solution.

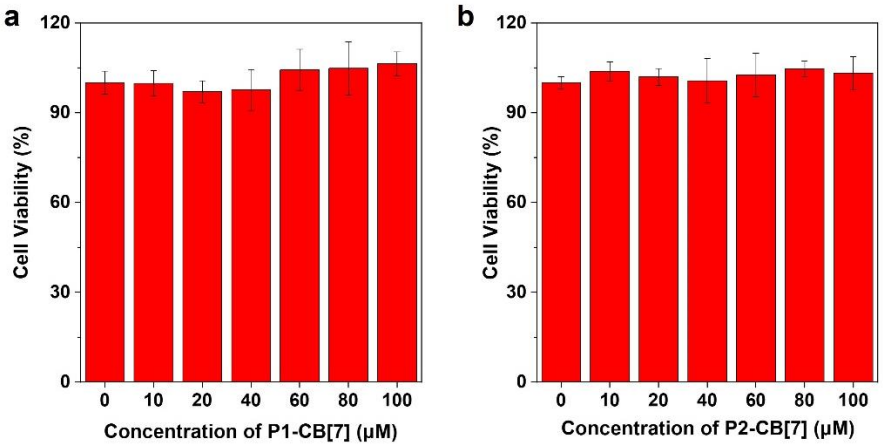



**Figure S15.** Cell viabilities of L929 cells treated with different concentrations of a) $P1^{\bullet+}$-CB[7] and b) $P2^{\bullet+}$-CB[7].

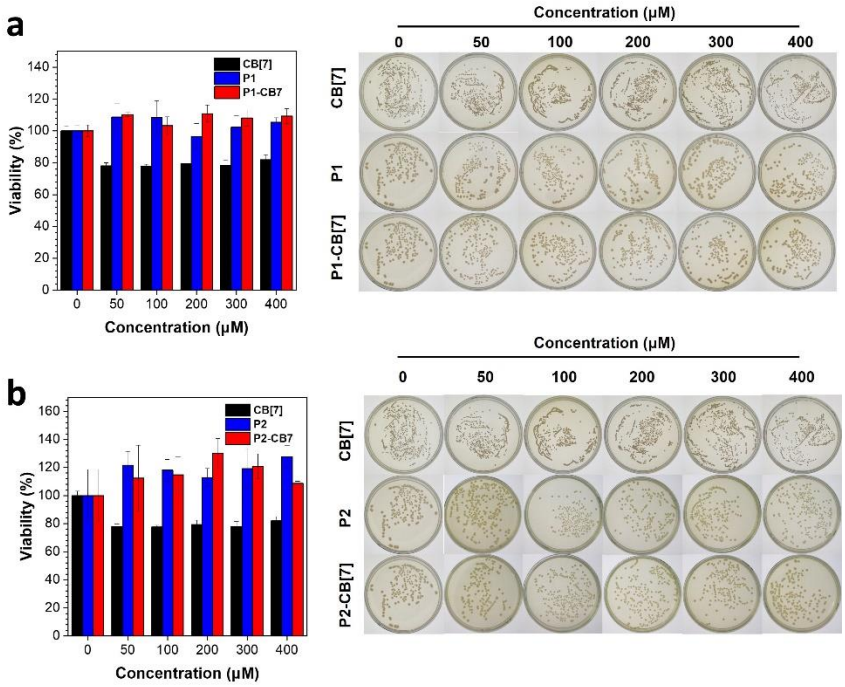



**Figure S16.** a, c) Bacterial viability and b, d) Photographs of agar plate of *E. coli* treated with different concentrations of CB[7], P1, P2, P1-CB[7], and P2-CB[7].

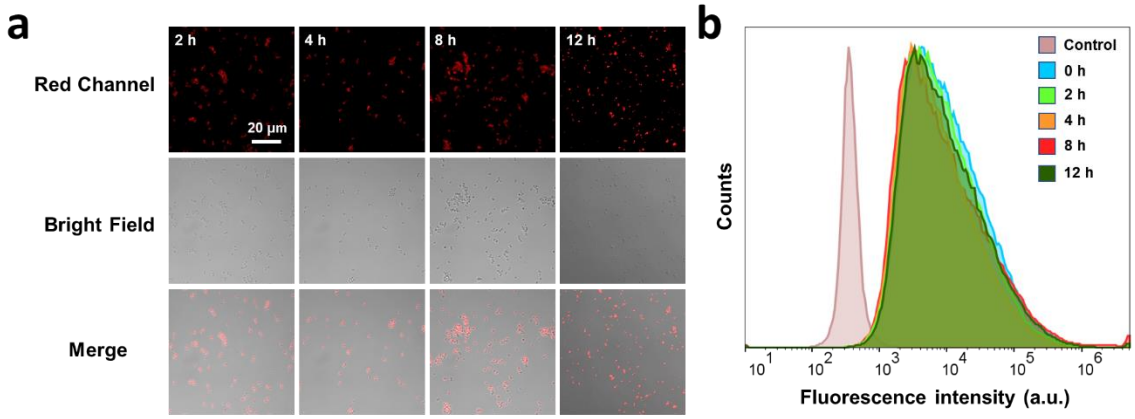



**Figure S17.** Time-dependent a) CLSM images and b) flow cytometric analysis of *S. aureus* treated with $P1^{\bullet+}$-CB[7]. For $P1^{\bullet+}$-CB[7], [P1] = 100 μM, [CB[7]] = 33.3 μM, $\lambda_{ex}$ = 633 nm, $\lambda_{em}$ = 650-750 nm.

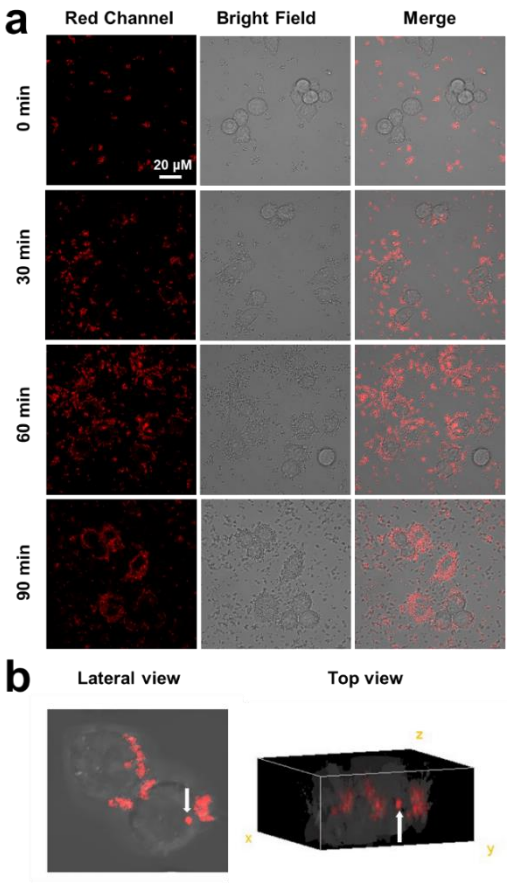



**Figure S18.** In situ monitoring of phagocytosis of *S. aureus* by macrophage RAW 264.7 cells: a) Time-dependent CLSM images. b) 3D CLSM images at 90 min. For $P1^{\bullet+}$-CB[7], [P1] = 100 μM, [CB[7]] = 33.3 μM, $\lambda_{ex}$ = 633 nm, $\lambda_{em}$ = 650-750 nm.

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