Supporting Information (SI)

pH-Triggered Nanoreactors as Oxidative Stress Amplifiers for Combating Multidrug-Resistant Biofilms

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MATERIALS AND METHODS

Chemical regents: Methanol, ethanol, anhydrous calcium chloride ($CaCl_2$), hydrogen peroxide aqueous solution (H_2O_2, 30%), polyethylene glycol (PEG)-200, ammonia solution (NH_3-H_2O), potassium iodide (KI) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ammonium molybdate tetrahydrate, dopamine hydrochloride were provided by Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Silver nitrate ($AgNO_3$) was obtained from Lingfeng Chemical reagent Co., Ltd. (Shanghai, China). 2′, 7′-dichlorofluoresceindiacetate (DCFH-DA) was obtained from Beyotime Biotechnology Co., Ltd. (China). 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (MCLA) was purchased from Chemical Industry Co., Ltd. (Tokyo, Japan).

Synthesis of CaO_2 nanoparticles (NPs): CaO_2 NPs were synthesized according to the previously reported method.[1] Briefly, $CaCl_2$ (3 g) was dissolved in deionized water (30 mL), and followed by the addition of PEG-200 (120 mL), and then stirred at 25 °C for 10 min. Subsequently, 1.5 mL H_2O_2 solution (30%) and 1.5 mL ammonia solution (28%) were added into the mixture with stirring, and the reaction was carried out for 2 h at room temperature. The mixture was centrifuged (8000 rpm, 15 min) and washed with 60 mL of methanol for 3 times. Finally, the CaO_2 NPs were obtained and kept in refrigerator before use.

Synthesis of polydopamine (PDA) coated CaO_2 nanoparticles (CP): Polydopamine (PDA) coated CaO_2 nanoparticles (CP) were synthesized *via* the oxidative self-polymerization of dopamine under alkaline conditions. In brief, 243 mg of the above-obtained CaO_2 NPs was uniformly dispersed in methanol (100 mL). Dopamine

hydrochloride (100 mg) was added to the suspension under stirring at room temperature for 12 h. The resultant products were collected by centrifugation (8000 rpm, 15 min) and washed with methanol (100 mL) for 3 times.

Synthesis of silver nanoparticle-doped, polydopamine (PDA)-coated CaO_2 nanoparticles (CPA): For synthesis of CPA NPs, 20 mg of CP was dispersed in 14 mL deionized water and 1 mL of ammonia solution (28%) under stirring at room temperature for 20 min. Then, 2 mL of $AgNO_3$ solution (10 mg mL^{-1}) was added, and the reaction was carried out under continuous stirring for 1 h. To completely reduce the Ag ions to Ag NPs, 2 mL of dopamine hydrochloride aqueous solution (1 mg mL^{-1}) was added into the above mixture and continuously stirred for 30 min at room temperature. The mixture solution was centrifuged (8000 rpm, 15 min) and washed 3 times with methanol, and finally dispersed in ethanol.

Synthesis of silver-modified polydopamine nanoparticles (PA): PDA nanoparticles were prepared according to the previously reported method.[4] Briefly, 10 mg of dopamine hydrochloride (Aladdin Chemical Co., China) and 100 μL of 1N NaOH solution were dissolved in 10 mL of deionized water, and stirred at room temperature. After stirring for 5 h, a dark brown mixture was obtained. Then PDA nanoparticles were collected by centrifugation (8000 rpm, 15 min) and washed with deionized water 3 times. For synthesis of PA nanoparticles, 10 mg of PDA was dispersed in 7 mL deionized water and 0.5 mL of ammonia solution (28%) under stirring at room temperature for 20 min. Then, 1 mL of $AgNO_3$ solution (10 mg mL^{-1}) was added, and the reaction was carried out under continuous stirring for 1 h. The mixture was

centrifuged (8000 rpm, 15 min) and washed 3 times with methanol. Finally, the PA nanoparticles were re-dispersed in ethanol.

Characterizations: The morphological images of NPs were acquired using a transmission electron microscope (HITACHI H-7000FA, HITACHI, Japan). The energy-dispersive X-ray spectroscopy (EDS) mapping analysis was characterized by HRTEM (FEI Talos-F200s, USA). The X-ray photoelectron spectroscopy (XPS) spectra of CPA were acquired using an X-ray photoelectron spectrometer (K-Alpha, Thermo Scientific, USA) using Al Kα radiation. The zeta potential and size distribution of NPs in aqueous solution were determined by dynamic light scattering (DLS, NanoZS ZEN3600, Malvern Instruments, UK) at 25 °C. The average diameter of particles was determined by using Image J software. The UV-vis absorption spectra of NPs were characterized by a UV-1800 spectrophotometer (Shimadzu, Japan). The FTIR spectra of NPs were recorded using a FTIR spectroscopy (Nexus, Thermal Nicolet, USA).

H_2O_2 generation from CPA: The *in vitro* H_2O_2 generation from nanoparticles was elvaluated as follows. Briefly, CaO_2 (0.7 mg), CP, and CPA (equivalent to 0.7 mg CaO_2) were suspended in 2 mL phosphate buffered saline (PBS) with two different pH values (7.0 and 5.5), and were incubated at 37 °C. At predetermined time points, 100 μL of release media was taken out and replaced with fresh media. The amount of generated H_2O_2 was determined by the potassium iodine reduction method,[2] in which I ions are oxidized to iodine generating the brown colored complex by H_2O_2 in presence of ammonium molybdate. Briefly, 20 μL of supernatant was mixed with 40 μL of KI solution (0.1 M) and 2 μL of ammonium molybdate solution (0.1 M), followed by

analyzing by an UV-vis spectrophotometer (UV1800, Shimadzu, Japan) at 352 nm after incubation at 37 ℃ for 30 min.

Determination of the ROS generation: DCFH-DA was utilized as a probe to determin the ROS production by measuring the fluorescence of DCF.[3] To test the ROS production capacity, CaO_2, free H_2O_2, CP, PA, and CPA (equivalent to 50 μg mL^{-1} of CaO_2) were mixed with DCFH-DA (final concentration of 10 μM) probe in phosphate buffered saline (PBS) with two different pH values (7.0 and 5.5). After 15 min, the DCF fluorescence intensity was measured by an EnSpire multimode plate reader (PerkinElmer, USA) every 10 min (excitation wavelength: 488 nm; emission wavelength: 525 nm).

For detecting the intracellular ROS content of *MRSA* and Amp ^{r} *E.coli* induced by different samples, both bacterial cells were treated with PBS, free H_2O_2, CaO_2, CP, PA, and CPA (equivalent to 50 μg mL^{-1} of CaO_2) at 37 °C for 2 h. Then these cells were incubated with DCFH-DA (the final concentration of 10 μM) probe for 30 min at 37 °C under dark. Finally, the DCF fluorescence intensity was analyzed by an EnSpire multimode plate reader (PerkinElmer, USA) (excitation wavelength: 488 nm; emission wavelength: 525 nm).

Determination of the production of $O_2^{\bullet-}$: MCLA was utilized as a chemiluminescence (CL) probe for measuring $O_2^{\bullet-}$ according to the CL-based MCLA method.[4] To test the formation of $O_2^{\bullet-}$, CaO_2, free H_2O_2, CP, PA, and CPA (50 μg mL^{-1} of CaO_2) were mixed with MCLA (the final concentration of 0.85 μM) in 100 μL of PBS at different pH values (7.0 and 5.5). After that, the mixtures yield superoxide specific CL that was

analyzed by an EnSpire multimode plate reader (PerkinElmer, USA) every 5 min. The formation of superoxide induced by CPA-1, CPA-2, and CPA-3 (equivalent to 50 μg mL^{-1} of CaO_2) was measured as described above.

For detecting the intracellular $O_2^{\bullet-}$ content of *MRSA* and Amp ^{r} *E.coli* induced by different agents, both bacterial cells were treated with PBS, free H_2O_2, CaO_2, CP, PA, and CPA (equivalent to 50 μg mL^{-1} of CaO_2) at 37 °C for 2 h. Thereafter, these cells were incubated with MCLA probe with final concentration of 0.85 μM for 3 min in the dark. At predetermined time intervals, the production of $O_2^{\bullet-}$ was analyzed by EnSpire multimode plate reader (PerkinElmer, USA).

Stability assay: In order to determine the stability of CPA in aqueous solution, the Ca^{2+} release profile was examined using a calcium colorimetic assay kit (Beyotime, China). Briefly, CaO_2 (0.7 mg), CP, and CPA (equivalent to 0.7 mg CaO_2) were dispersed in 2 mL PBS with two different pH values (7.0 and 5.5), and were incubated at 37 °C. At predetermined time points, 100 μL of release media was taken out and replaced with fresh media. The amount of generated Ca^{2+} was determined using a calcium colorimetic assay kit according to the manufacturer's instructions.

Silver ions release behavior: CPA and PA NPs were suspended in 1 mL of PBS with two different pH values (7.0 and 5.5), and incubated at 37 °C. At predetermined time points, 100 μL of released media was taken out and replaced with fresh media. The obtained samples were centrifuged at 12,000 rpm for 5 min, then the amout of the released Ag ions were measured using flame atomic absorption spectrometry (FAAS).

Bacterial culture and antibacterial experiments: Methicillin resistant *Staphylococcus aureus* (*MRSA*), ampicillin resistant *Escherichia coli* (Amp ^{r} *E.coli*) were obtained from Clinical Laboratory of Union Hospital (Wuhan, China). All bacteria were cultured at 37 °C with shaking at a speed of 200 rpm in fresh liquid LB medium overnight. When the OD600nm (optical density measured by a microplate reader at a wavelength of 600 nm) of bacterial suspension reach 0.1, the bacteria were diluted with liquid LB medium (1:10^4 dilution). The antibacterial activity was elvaluated using colony counting assay as follows. 100 μL of the as-prepared bacteria suspension was mixed with PBS, free H_2O_2, CaO_2, PA, CP, and CPA at different concentrations for 30 min. Then the mixtures were driped on solid Mueller-Hinton Broth and dispersed by spread plate method. After incubating for 24 h, the viability was determined by counting the number of the colony-forming units on the plates.

Bacterial antibiotic-resistance assay: Single colony of standard strains of Staphylococcus aureus (ATCC25923), *MRSA*, standard strains of Escherichia coli (ATCC25922), and Amp ^{r} *E.coli* were taken in fresh LB liquid medium respectively, and cultured at 37 °C with shaking at a speed of 200 rpm. When the OD600nm of bacterial suspension reached 0.1, 100 μL of the bacteria suspension was spread on solid Mueller-Hinton Broth respectively. Corresponding plates with cefoxitin (FOX) and ampicillin (AMP) were put in the middle of the solid Mueller-Hinton Broth. After incubation at 37 °C for 24h, the diameters of the bacteriostatic rings were measured and photographed.

Calcium content detection: The amount of the calcium in bacteria treated with CPA was determined *via* the calcium colorimetic assay kit (Beyotime, China). 100 μ L of Amp ^{r} *E. coli* and *MRSA* cells were added into a 1.5 mL eppendorf tube and incubated with PBS, CaO_2, CP, and CPA (equivalent to 50 μg mL^{-1} of CaO_2) at 37 °C for 2 h under shaking at a speed of 200 rpm. Subsequently, the cells with different treatment were collected by centrifugation (5000 rpm, 5 min) and washed with PBS for 3 times. Thereafter, the calcium concentration was assessed *via* the calcium colorimetic assay kit according to the manufacturer's instructions. In addition, the cell lysates were quantified by BCA protein assay kit (Bio-Rad, USA).

Detection of association of NPs with MRSA: The association (contact and uptake) of NPs with bacteria was determined by TEM and flow cytometry using the side-scatter signal according to previous reports.[5, 6] A single *MRSA* bacteria colony was taken in the fresh LB liquid medium and cultured at 37 °C with shaking at a speed of 200 rpm. When the OD600nm of bacterial suspension reached 1.0, 1 mL bacterial suspension was taken out and centrifuged at 5000 rpm for 5 min to collect bacteria. Then the *MRSA* incubated with PBS, CaO_2, CP, and CPA (equivalent of 50 μg mL^{-1} CaO_2) at 37 °C for 1 h under shaking at a speed of 200 rpm. Subsequently, these bacteria were collected by centrifugation (5000 rpm, 5 min), washed with PBS for 3 times, and resuspended in PBS containing 2.5 % glutaraldehyde at 4 °C for 4h, followed by flow cytometry analysis and TEM observation after being negative staining with sodium phosphotungstate, respectively.

In vitro antibiofilm assay: The biofilms of *MRSA* were allowed to form in 24-well microplates. Briefly, 10 μL of *MRSA* seed suspensions and 990 μL of LB medium were added into each well of 24-well microplates and incubated without shaking at 37 °C for 24 h. The medium was replaced with fresh medium and cultured for 48 h to estabilish mature biofilm. Then, the supernatants were removed and washed with PBS buffer under aseptic condition, followed by incubating 1 mL of fresh LB medium containing PBS, free H_2O_2, CaO_2, CP, PA, and CPA (equivalent to 50 μg mL^{-1} of CaO_2), respectively. After 12 h incubation, the medium was removed and washed gently with 1.0 mL PBS. And the remaining biofilms were stained by 300 μL of 1.0% crystal violet (CV) solution for 10 min, followed by washing twice with 1 mL PBS and drying at room tempreture. Subsequently, 0.5 mL of 95% ethanol was added to each well and incubated 37 °C for 1 h. The extraction solutions of CV dye were measured by a microplate reader at 595 nm.

For confocal microscopy (CM) imaging analysis, the biofims were cultured on sterile glass slides and treated with PBS, free H_2O_2, CaO_2, CP , PA, and CPA (equivalent to 50 μg mL^{-1} of CaO_2), respectively, as described above. The biofilms were washed twice with 1mL of PBS and then stained using live/dead staining kit according to the manufacturer's instructions (Biovision, CA, USA). Finally, the biofilms were imaged using a confocal microscopy system (Revolution XD, Andor).

In vitro cytotoxicity evaluation: Mouse myoblast cells (C2C12, obtained from ATCC) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin and

100 μg mL^{-1} streptomycin and incubated at 37 °C in a humidified incubator with 5% CO_2. The cells were seeded in 96-well plates at a density of around 10^4 cells per well and treated with H_2O_2, CaO_2, CP, PA, and CPA at various concentrations (equivalent to 10, 20, 40, and 60 μg mL^{-1} of CaO_2) under two different pH values (7.0 and 5.5) for 24 h. The cytotoxicity was assessed using a Cell Counting Kit-8 (CCK-8, Dojindo laboratories, Japan) assay according to the manufacturer's instructions. Cell viability within each group was determined as a percentage of the viability of PBS-treated cells.

Statistical analysis: Each experiment was performed at least three times. Comparisons between two groups were analyzed using Student's *t*-test. All date was presented as mean ± SD. Statistical significance was set at $*p < 0.05$, $**p < 0.01$ and *N.S.*, not significant.

SUPPLEMENTARY RESULTS

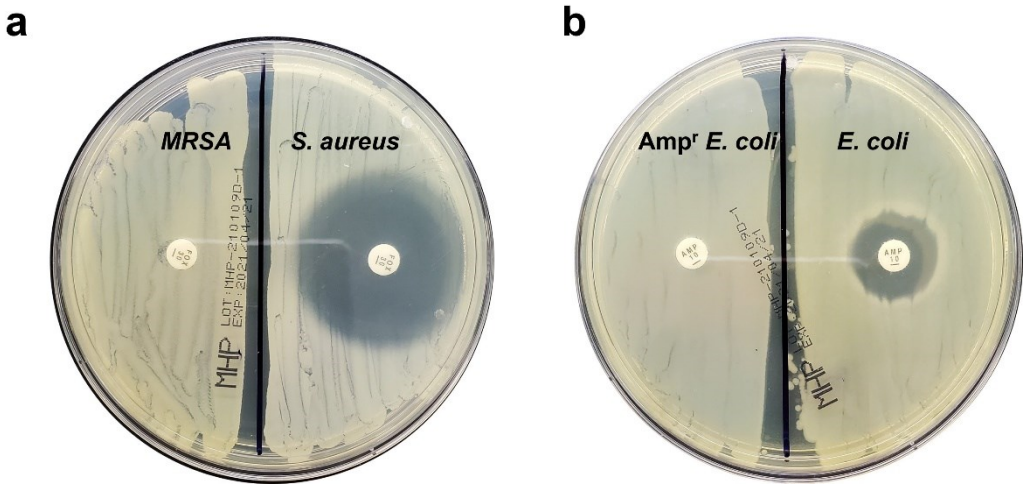

Fig. S1 Detections of methicillin and ampicillin resistance in (a) *MRSA* and (b) Amp ^{r} *E. coli* respectively, using disk diffusion assays. Detecting inhibition zones of *S. aureus* and *MRSA* with cefoxitin (FOX) plates at the same concentration, and *E. coli* and Amp ^{r} *E. coli* with ampicillin (AMP) plates at the same concentration, respectively.

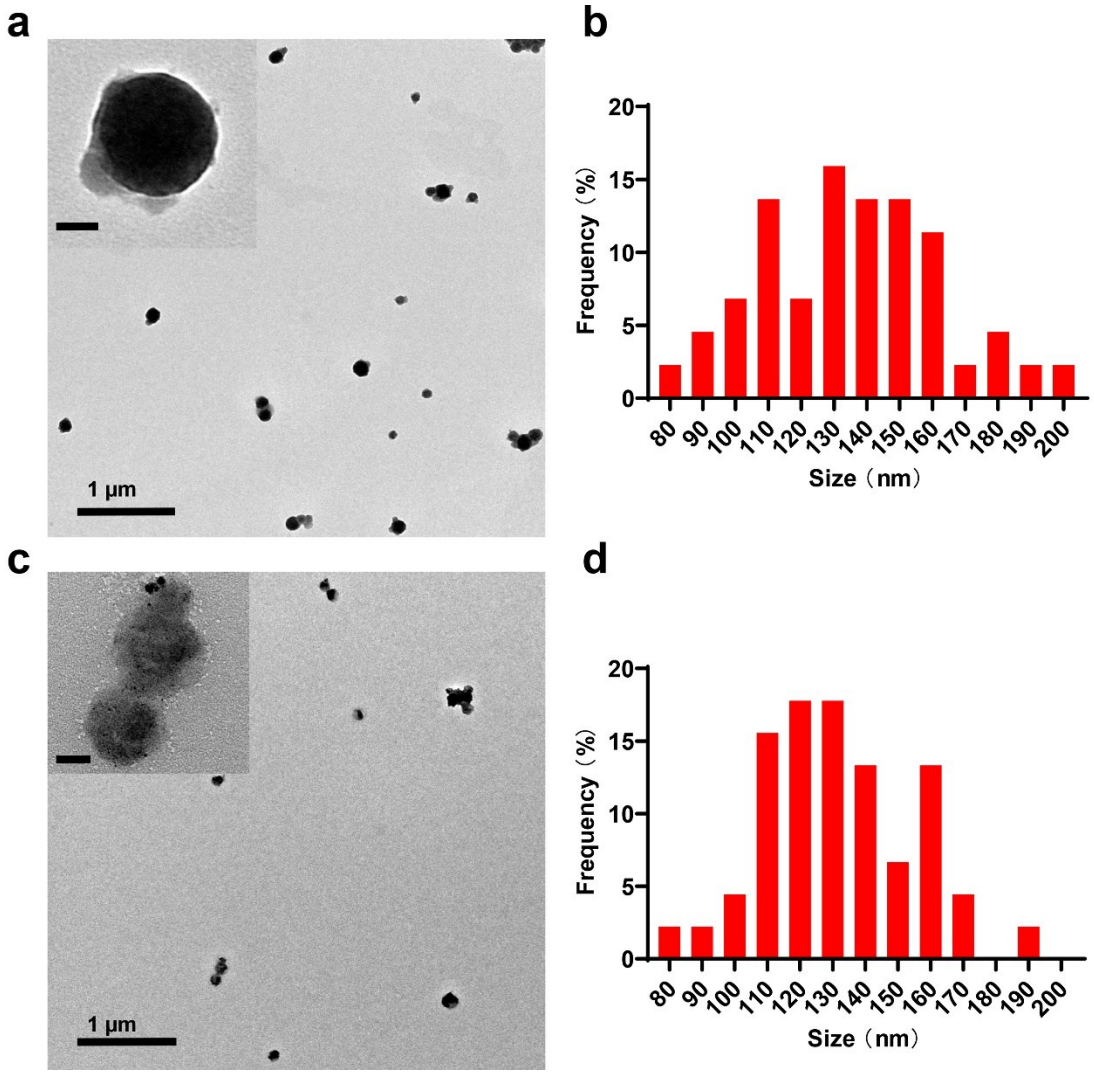

Fig. S2 The low-magnification TEM images of (a) CP and (c) CPA. Size distribution histograms of (b) CP and (d) CPA.

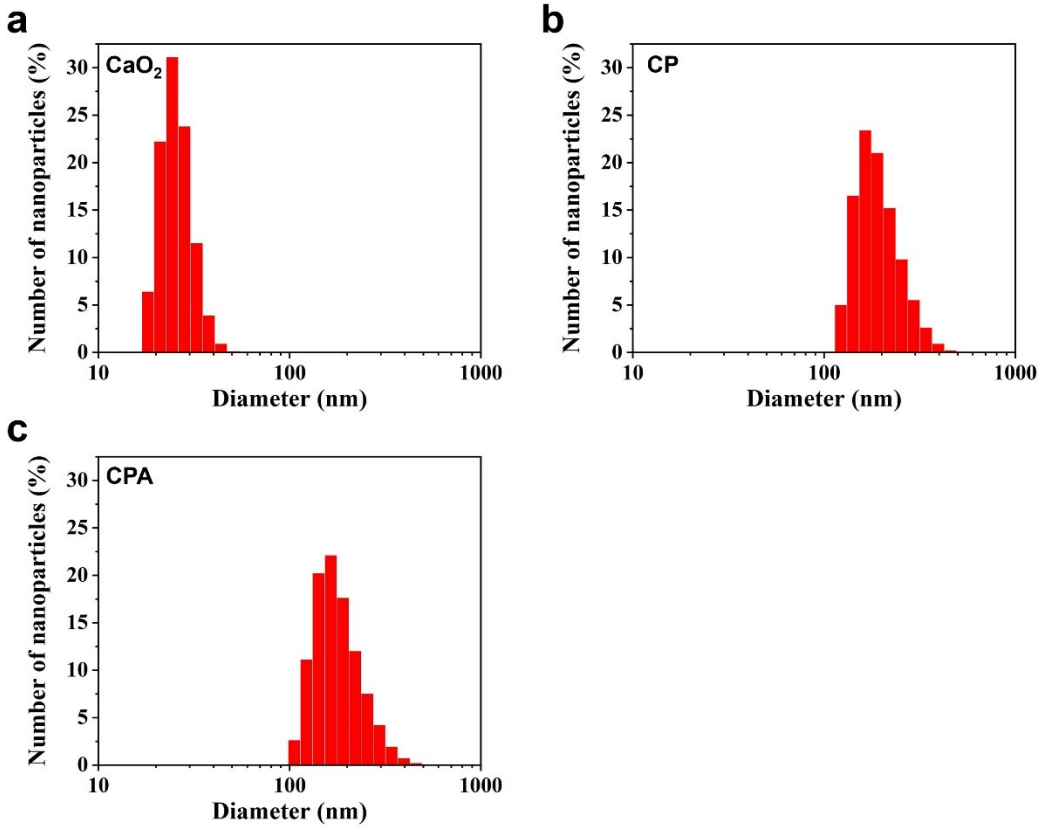

Fig. S3 Size distribution of (a) CaO_2, (b) CP, and (c) CPA in aqueous solution was analyzed by dynamic light scattering (DLS).

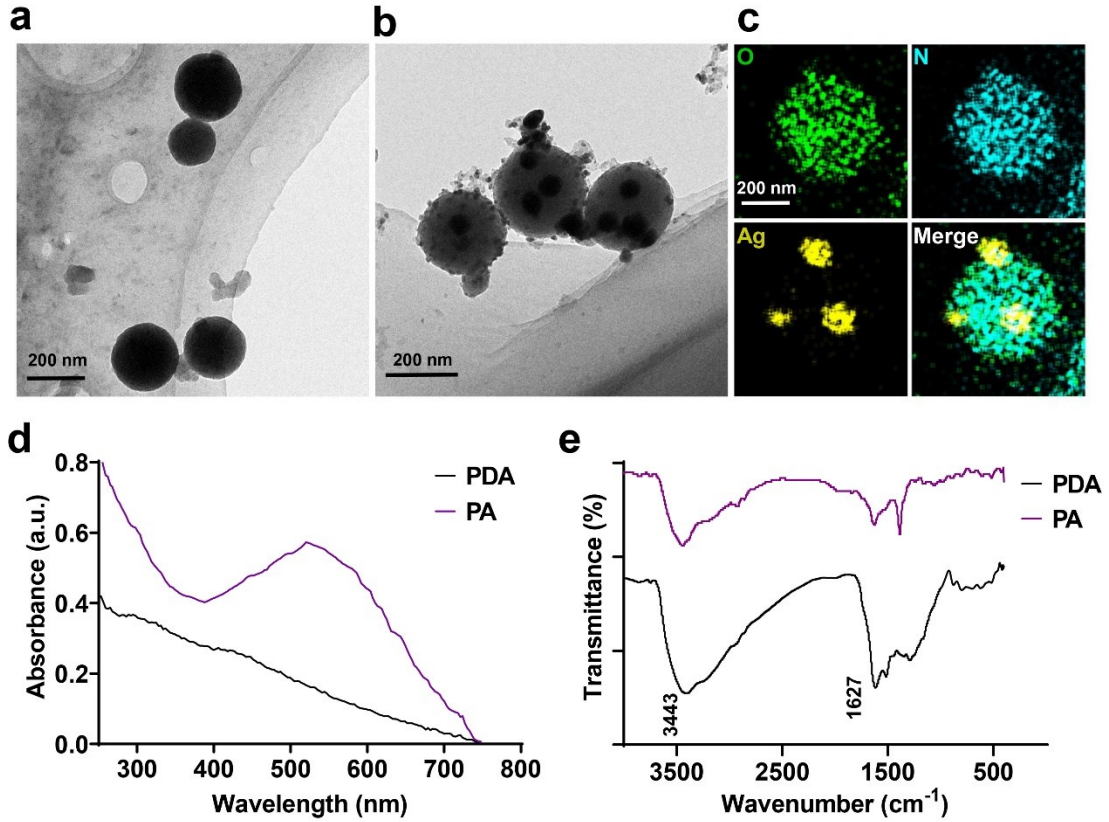

Fig. S4 Characterizations of PA. The representative TEM images of (a) PDA and (b) PA, respectively. (c) EDS elemental mapping of O, N, and Ag in PA. (d) UV-vis absorbance spectra and (e) FTIR spectra of PDA and PA.

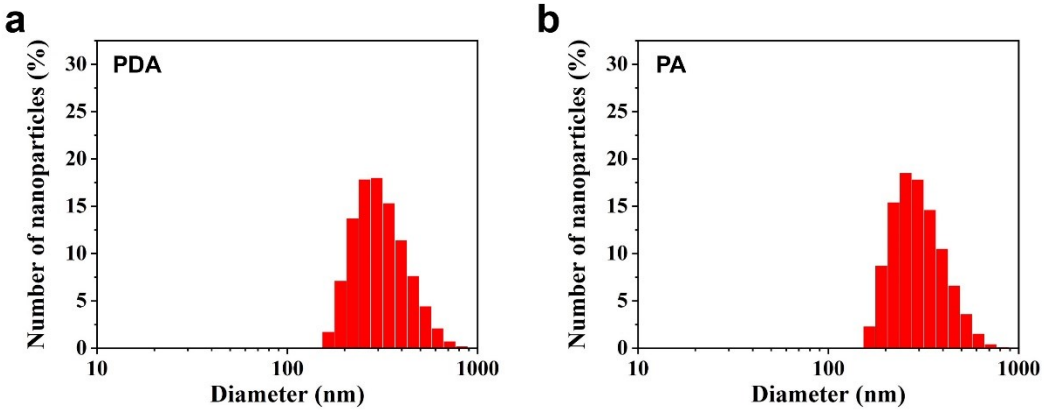

Fig. S5 Size distribution of (a) PDA and (b) PA in aqueous solution was analyzed by DLS.

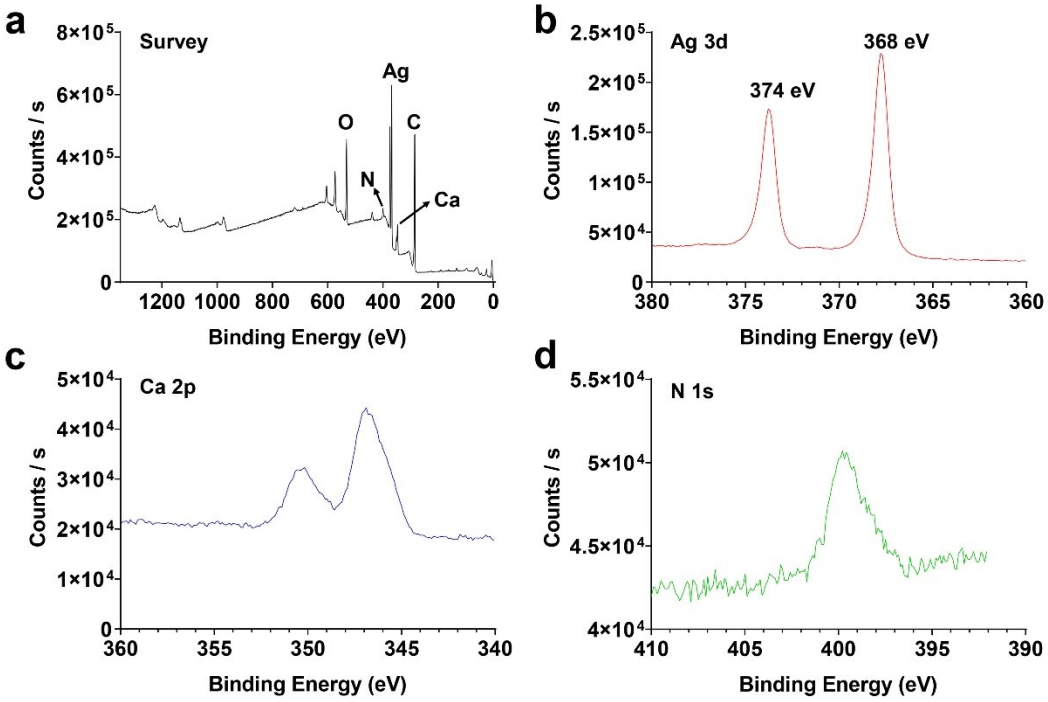

Fig. S6 The survey XPS spectrum of (a) CPA. XPS high-resolution spectrum of (b) Ag, (c) Ca, and (d) N in CPA, respectively.

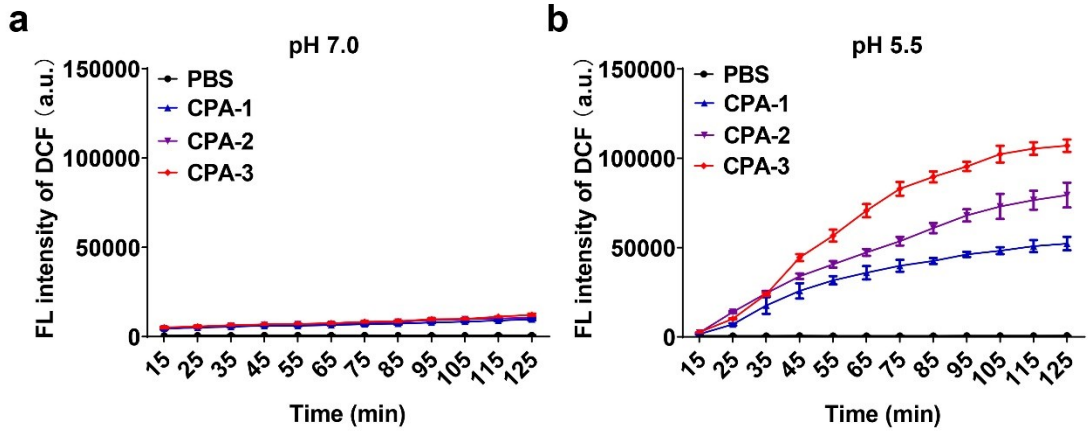

Fig. S7 Production of ROS from different agents at (a) pH 7.0 and (b) pH 5.5 determined by measuring the fluorescence intensity of DCF. CPA-1, CPA-2, and CPA-3 were fabricated *via* the *in situ* reduction of silver ions on the surface of CP with the concentrations of $AgNO_3$ solution at 2, 6, and 10 mg mL^{-1}, respectively.

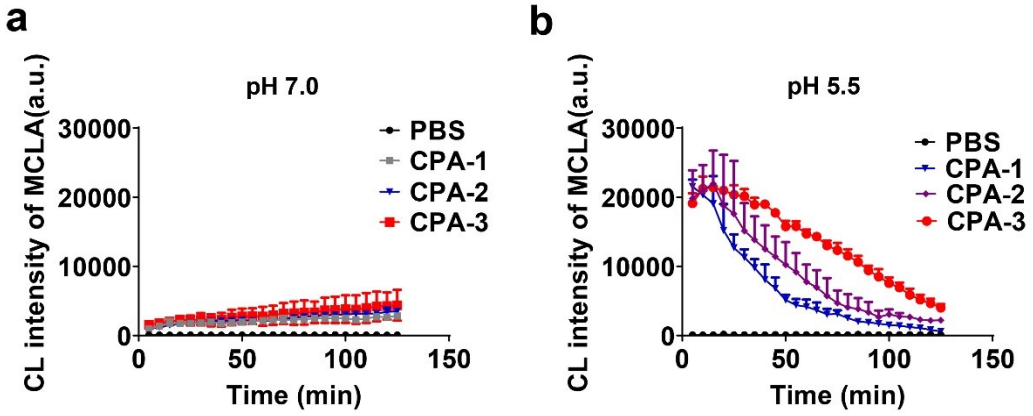

Fig. S8 Production of $O_2^{\bullet-}$ from different agents at (a) pH 7.0 and (b) pH 5.5. The generation of $O_2^{\bullet-}$ was determined by measuring the CL intensity of MCLA.

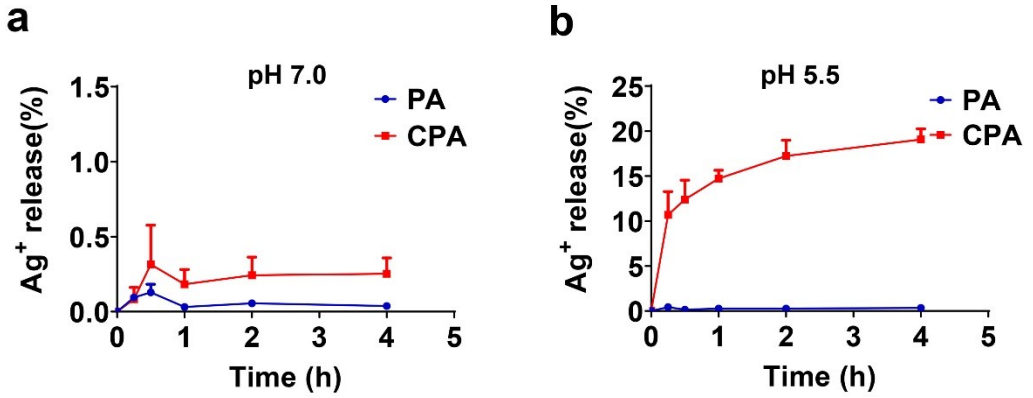

Fig. S9 The Ag ions release behavior of CPA *in vitro*. Release profiles of Ag ions from PA and CPA at (a) pH 7.0 and (b) pH 5.5.

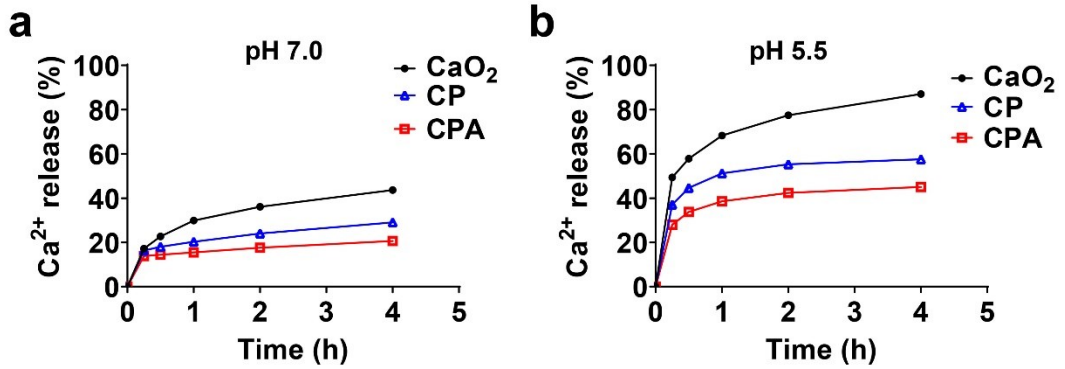

Fig. S10 Release profiles of Ca^{2+} from CaO_2, CP, and CPA dispersed in PBS at (a) pH 7.0 and (b) pH 5.5.

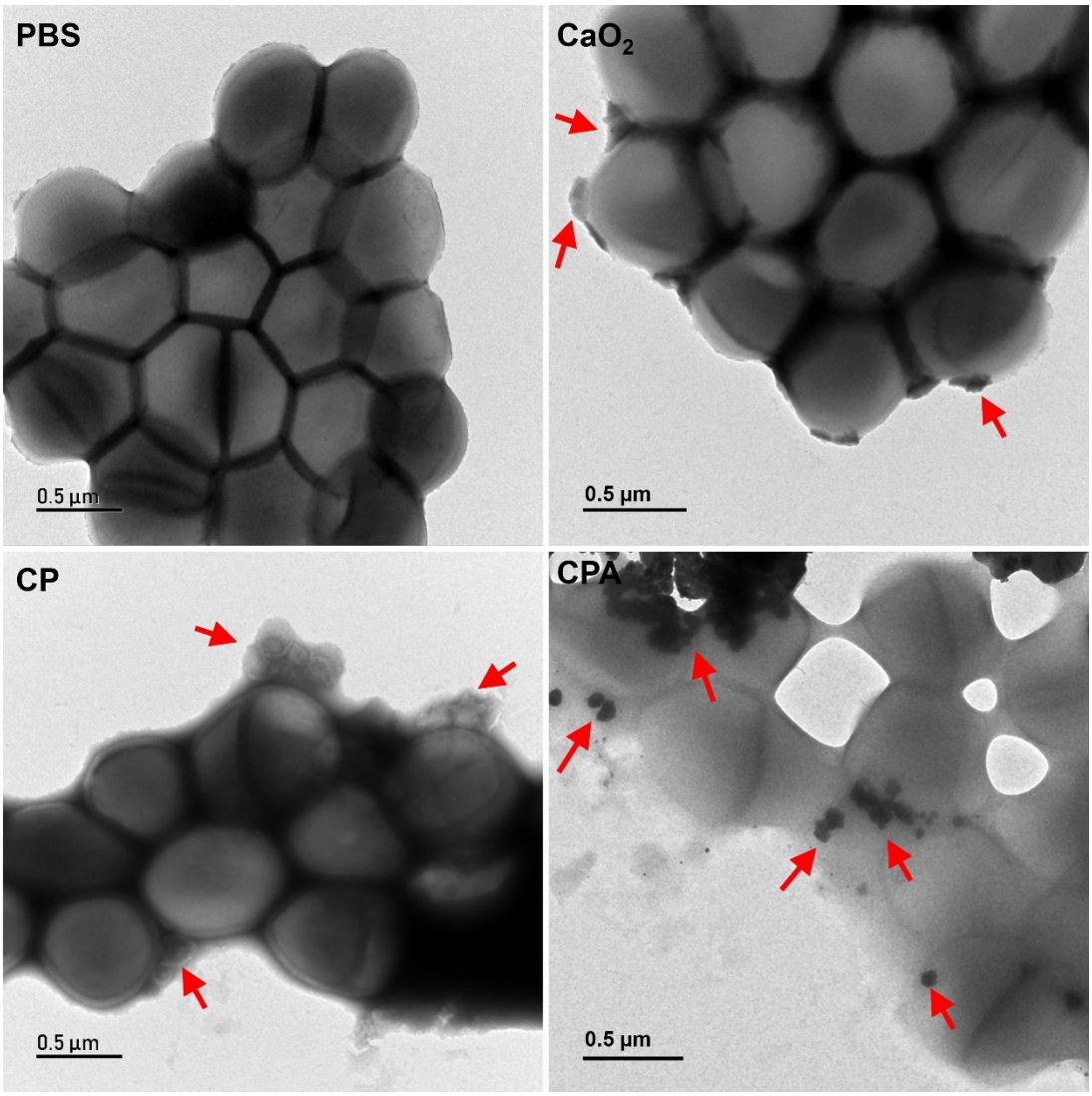

Fig. S11 TEM images of *MRSA* incubated with PBS, CaO_2, CP, and CPA for 1 h. The nanoparticles were observed on the surface of the bacterial cells (red arrows).

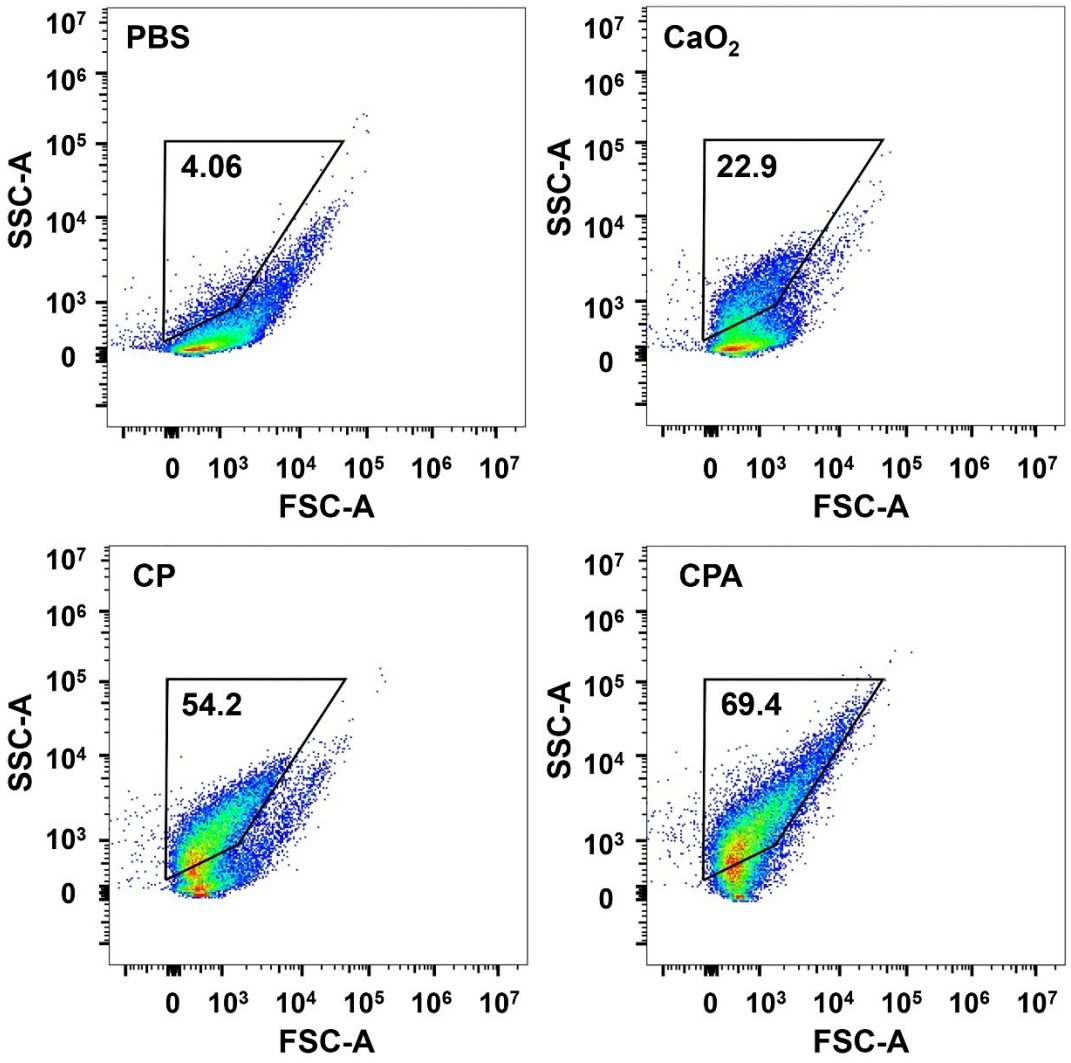

Fig. S12 Scatter analysis of the *MRSA* incubated with PBS, CaO_2, CP, and CPA for 1 h by flow cytometry.

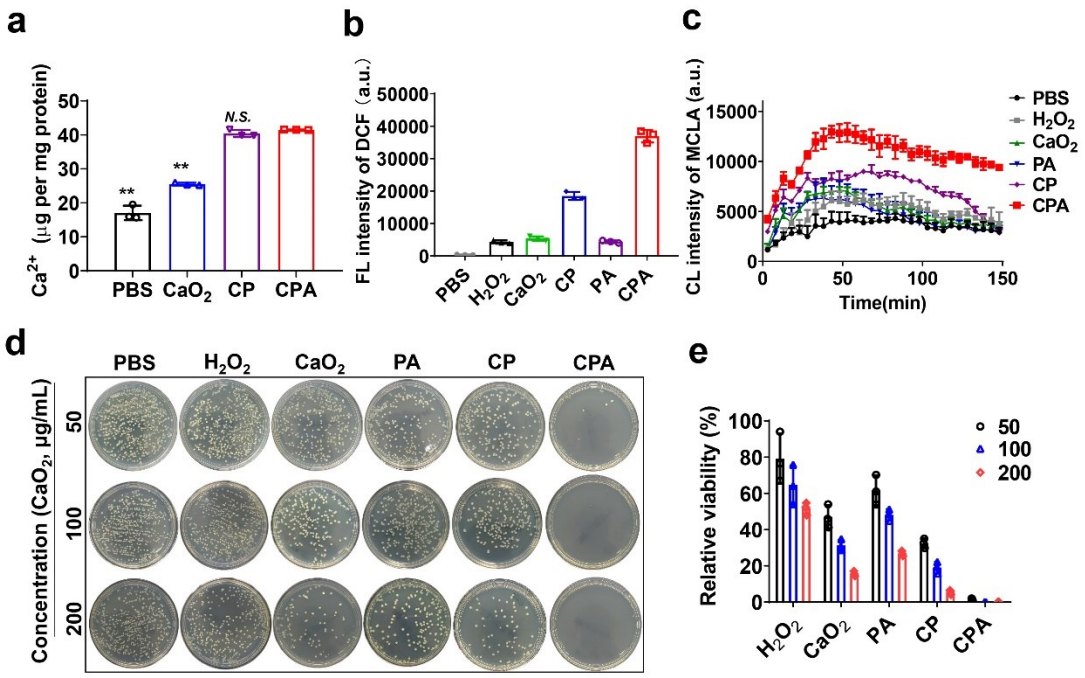

Fig. S13 The antibacterial effect of CPA against Amp ^{r} *E. coli*. (a) The calcium in Amp ^{r} *E. coli* incubated with PBS, CaO_2, CP, and CPA for 2 h. The intracellular (b) ROS and (c) $O_2^{\bullet-}$ in Amp ^{r} *E. coli* treated with PBS, free H_2O_2, CaO_2, PA, CP, and CPA for 2 h. The final concentrations of CaO_2, CP, and CPA used in those experiments were equal to 50 μg mL^{-1} of CaO_2. (d) The representative photographs of agar plates of Amp ^{r} *E. coli* treated with PBS, free H_2O_2, CaO_2, PA, CP, and CPA at different concentrations. (e) The relative viabilities of Amp ^{r} *E. coli* after treatment with PBS, free H_2O_2, CaO_2, PA, CP, and CPA at different concentrations were evaluated by colony-forming units assay. Data shown as mean ± SD; $^{**}p < 0.01$; *N.S.*, not significant.

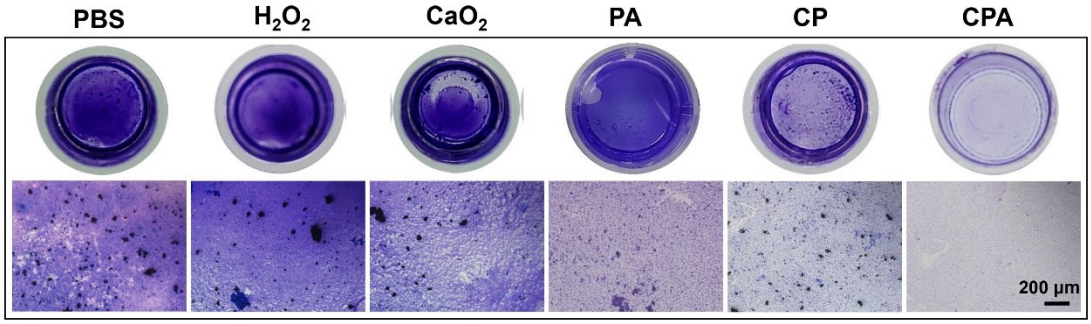

Fig. S14 The representative photographs of crystal violet stained *MRSA* biofilms after treatment with PBS, free H_2O_2, CaO_2, PA, CP, and CPA. Scale bar, 200 μm.

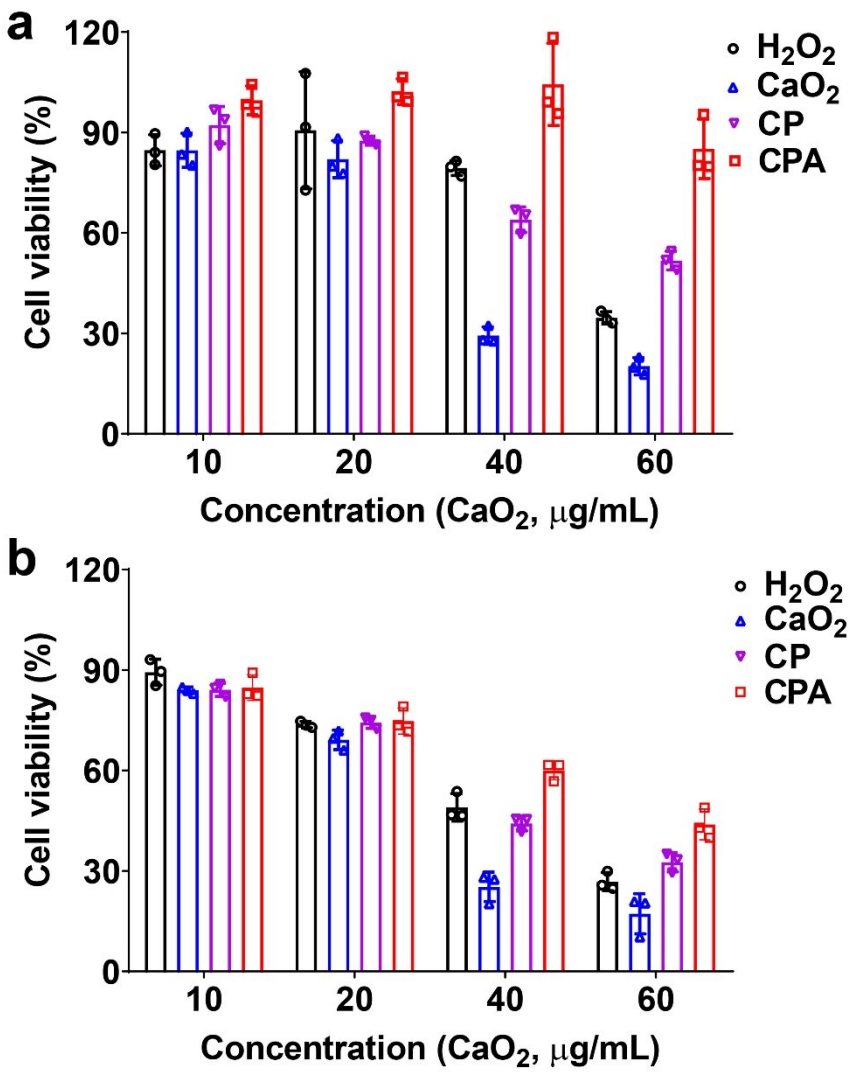

Fig. S15 Cytotoxicity assays of CPA against mouse myoblast cells (C2C12) in cell culture media at (a) pH 7.4 and (b) 5.5 for 24 h. Data shown as mean ± SD.

Table S1. The content of each component in various nanoparticle formulations.

Formulation	Content (%)		
	CaO_2 [a)]	PDA	Ag [b)]
CPA	65.5	30.2	4.3
CP	70.9	29.1	-
PA	-	81.7	18.3

[a)] The amount of CaO_2 from each formulation was measured using the calcium assay kit.

[b)] The Ag content was determined by flame atomic absorption spectrometry (FAAS).

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