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Supporting Information

A Fluorescent Nanobiocide Based on ROS-generation for Eliminating Pathogenic and Multi-drug Resistant Bacteria

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Experimental Methods

1. Reagents

Copper(I) iodide (CuI), zinc stearate ($Zn(SA)_2$), 1-dodecanethiol (DDT), oleylamine (OAm), oleic acid (OA), 1-octadecene (ODE), and zinc acetate ($Zn(OAc)_2$) were purchased from Aladdin (Shanghai, China). Indium (III) acetate ($In(OA)_3$) and 2,2′-azobisisobutyronitrile (AIBN), which needed to be recrystallized and dried in vacuum before use, were obtained from Sigma-Aldrich (St.Louis). α-D-Galactose pentaacetate, and 2,7-dichlorofluorescein diacetate (DCFH-DA) were brought from Tianjin Heowns Biochem Technologies LLC (Tianjin, China). Fucose was obtained from Chemlin Chemical Company (Nanjing, China). The chain transfer agent, 4-cyanopentanoic acid dithiobenzoate (CPADB), was synthesized according to the previous method.[1]

2. Preparation of ZCIS QDs

On the basis of the previously reported method,[2] Zn stock solution was primarily prepared. Briefly, 0.25 mmol of $Zn(OAc)_2$, 4 mL of ODE, and 1 mL of OAm were mixed in a three-necked flask. After being degassed under vacuum for several times, the system was heated to 160 ℃ under nitrogen and reacted for 10 min until the solution became clear. Then, the mixture of CuI (0.1 mmol), $In(OAc)_3$ (0.1 mmol), DDT (1 mL), $Zn(SA)_2$ (0.05 mmol), OA (0.1 mL) and ODE (4 mL) in three-necked flask was degassed for 20 min at 120 ℃. The colloidal solution was formed after reacting for 30 min at 230 ℃ with the protection of nitrogen. When the temperature decreased to 150 ℃, 5 mL Zn stock solution was added into the flask dropwise and reacted for 30 min at 230 ℃. The reaction was terminated by chloroform, and gradually reduced to room

temperature. Subsequently, the system was purified with acetone and centrifuged at 8, 000 rpm for 5 min. Two more purifications with a certain percentage of hexane and ethanol to centrifugation were essential. Finally, the obtained ZCIS QDs were dispersed in chloroform at 4 ℃.

3. Synthesis of 2-(β-D-galactosyloxy)ethyl methacrylate (GEMA).

2-Methacryloyloxyethyl-(2,3,4,6-tetraace-tyl-β-$_D$-galactopyranoside) (AcGEMA) was synthetized according to the previous method.[3] Briefly, boron trifluoride ethylether (7 mL, 44 mmol) was dropwise added into the dried dichloromethane (20 mL), mixing galactose pentacetate (3.9 g, 10 mmol) and 2-hydroxyethyl methacrylate (2.6 mL, 20 mmol), then reacted at 0 °C under nitrogen for 1 h. The reaction was transferred to room temperature and further reacted for 3 h, followed by adding chloroform (60 mL) for quenching. The combined organic phases were washed successively with saturated aqueous $NaHCO_3$ and brine. After drying over Na_2SO_4, filtration and concentration, the colorless product was acquired. ^{1}H NMR ($CDCl_3$, ppm, Fig. S3): 5.64-5.57 (m, 1H), 6.13 (s, 1H), 5.39 (d, J = 2.6 Hz, 1H), 5.22 (dd, J = 10.4, 8.0 Hz, 1H), 5.01 (dd, J = 10.5, 3.4 Hz, 1H), 4.54 (d, J = 8.0 Hz, 1H), 4.38 - 4.24 (m, 3H),4.21-4.01 (m, 2H), 3.96-3.79 (m, 2H), 2.21-1.88 (m, 15H).

AcGEMA (500 mg, 1.1 mmol) was added into CH_3ONa/CH_3OH solution (20 mL, 2 mmol/L) under stirring at room temperature until there was not much residual unprotected material at the upper baseline on the chromatographic sheet. After being terminated by hydrochloric acid, 2-(*β*-D-galactosyloxy)ethyl methacrylate (GEMA) was separated by silica column chromatography with an eluent of CH_2Cl_2/CH_3OH

mixed solution (v/v, 4:1). ^{1}H NMR (D_2O, ppm, Fig. S3): 6.12 (s, 1H), 5.68 (s, 1H), 4.46-4.25 (m, 3H), 4.11 (dd, J = 10.7, 6.9 Hz, 1H), 3.94 (ddd, J = 12.1, 5.6, 3.7 Hz, 1H), 3.87 (d, J = 3.3 Hz, 1H), 3.73-3.56 (m, 4H), 3.47 (dd, J = 9.8, 7.9 Hz, 1H), 1.89 (s, 3H).

4. Synthesis of 2-(2′,3′,4′-L-Fucose)ethyl Methacrylate (FEMA)

According to the previous work,[3] the reaction system including the dried fucose (16.4 mg, 0.1 mmol) and anhydrous pyridine was accelerated by the dripped acetic anhydride (62.3 mg, 0.61 mmol). After 5 h of reaction, the organic system was purified by ethyl acetate, water, HCl solution, $NaHCO_3$ and anhydrous Na_2SO_4. Eventually, a lightly yellow crystal named L-fucose tetraacetate was prepared after concentration. The obtained product and hydroxyethyl acrylate (32.5 μL, 0.25 mmol) were dissolved in dried dichloromethane enclosed with nitrogen, and slowly added boron trifluoride ethylether (17.5 μL, 0.11 mmol) at 25 °C for 8 h reaction. After being quenched by chloroform (750 μL), the reaction system was washed with deionized water and $NaHCO_3$ solution, then dried and evaporated to acquire the final product named AcFEMA. ^{1}H NMR ($CDCl_3$, ppm, Fig. S3): 6.13 (s, 1H), 5.62-5.57 (m, 1H), 5.26-5.16 (m, 2H), 5.01 (dd, J = 10.4, 3.5 Hz, 1H), 4.51 (d, J = 7.9 Hz, 1H), 4.35-4.24 (m, 2H), 4.06 (ddd, J = 11.6, 5.0, 3.7 Hz, 1H), 3.82 (ddd, J = 6.4, 4.9, 2.4 Hz, 2H), 2.03 (dd, J = 50.6, 37.4 Hz, 12H), 1.22 (d, J = 6.4 Hz, 3H).

Similar to the deprotection method of AcGEMA, FEMA was obtained after the deprotection of AcFEMA by CH_3ONa/CH_3OH solution (20 mL, 2.5 mmol/L) was needed. ^{1}H NMR (D_2O, ppm, Fig. S3): 6.12 (d, J = 7.0 Hz, 1H), 5.72-5.68 (m, 1H), 4.41-4.30 (m, 3H), 4.09 (ddd, J = 12.1, 5.7, 3.2 Hz, 1H), 3.97-3.89 (m, 1H), 3.76-3.68

(m, 2H), 3.59 (dd, J = 9.9, 3.5 Hz, 1H), 3.44 (dd, J = 9.9, 7.9 Hz, 1H), 1.92 (d, J = 15.0 Hz, 3H), 1.26-1.13 (m, 3H).

5. Preparation of p(GEMA-*r*-FEMA)

The synthesis of p(GEMA-*r*-FEMA) was conducted by RAFT polymerization with a fixed ratio of monomer to chain transfer agent in DMF. After adding initiator AIBN, chain transfer agent CPADB, monomer GEMA and FEMA at ratios of 1:1:10:10, 1:1:20:20, and 1:1:30:30, the system was degassed by nitrogen for half an hour. After 24 h of reacting at 70℃, the mixture was quenched by ice. The resultant p(GEMA-*r*-FEMA) was isolated by dialysis (molecular weight cutoff, 3500 Da) against deionized water and lyophilization to get solvent-free products.

6. Preparation of ZCIS QDs@p(GEMA-*r*-FEMA) (QDs@pG/F)

Due to the hydrophobic property of QDs, the obtained ZCIS QDs were modified by hydrophilic polymers for further biological applications.

To get the thiol at the end of the chain, $NaBH_4$ solution (20 mg/mL) was added to the solution of polymers (1×10^{-3} mmol/mL) and the mixture was stirred at 25 ℃ for 15 min. After dialysis and cold drying, the polymers with sulfhydryl groups were added into 2 mL DMF with 2 mg QDs. The turbid solution was heated to 150 ℃ and stirred with the protection of nitrogen. The system became clear gradually within 10 min. Then the obtained product was purified by centrifugating at 10, 000 rpm for 5 min and repeated twice.

7. Characterization

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded using a Varian

Unity-plus 400 NMR spectrometer. The absorption spectra were recorded with the UV-vis spectrophotometer (Shimadzu 2250) and photoluminescence (PL) spectra were taken using the fluorescence spectrophotometer (Shimadzu RF-5301PC). The crystal structure of QDs was characterized by X-ray diffractometer (XRD) (Rigaku, D/max-2500 using a Cu tube). We used X-ray photoelectron spectroscopy (XPS, Axis Ultra DLD) to determine the chemical components. Morphological images were taken by a JEM-2100F transmission electron microscope (TEM) with an acceleration voltage of 200 kV. Hydrodynamic diameter (D_H) and size distribution (PDI) of nanoparticles were characterized by dynamic light scattering (DLS) using a Malvern Zetasizer Nano S apparatus equipped with a 4.0 mV laser operating at $\lambda = 636$ nm.

8. Bacterial Adherence Analysis

Four strains of *P. aeruginosa*, PA14, PAK, PAO1 and MDR PA, were used to evaluate the clustering effect of the nanobiocides. Three kinds of nanobiocides were incubated with *P. aeruginosa* (1.5×10^8 CFU/mL) for 3 h and bacteria clusters were centrifuged at 5000 rpm. After washing three times with PBS, bacteria clusters were attached to slides by glycomimetics. Then the adhesion effect was visualized under confocal laser scanning microscope (CLSM, Leica TCS SP8) at the maximum excitation of 660 nm and the emission wavelength of 730 nm.

To evaluate the impact of the material concentration on the adhesion effect, the absorptions of the supernatant from bacterial suspension were measured. Briefly, *P. aeruginosa* diluted to an appropriate concentration ($OD_{600} = 1.5$), and incubated equivalently with the nanobiocides in PBS at a series of concentrations (0, 16, 32, 63,

125, 250, and 500 μg/mL). Then the upper layer suspension of the mixed suspension was detected the absorption at 600 nm after 3 h of co-culture at 37 ℃. Next, we used the same method to probe the impact of acting time on adhesion effect and the above experiments were all conducted in triplicate.

9. Inhibition of Bacteria Binding to Host Cells

Aiming to determine the antiadhesion effect of the materials, we used mouse fibroblasts (NIH-3T3, ATCC CRL-1658) cells infected by *P. aeruginosa*, which were commonly found in a cystic fibrosis disease. The cells were seeded in a 24-well PVC microtiter plate containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and cultured for 12 h. To label bacteria, 2 mg of fluorescein isothiocyanate (FITC) was added into 22 mL of LB medium containing 2 mL of the original bacterium fluid (10^9 CFU/mL) and incubated in the table concentrator overnight, then the FITC-labeled *P. aeruginosa* were obtained. Then 80 μL of FITC-labeled *P. aeruginosa* suspension (OD_{600} = 1.5) was added into each well. After 0.5 h of the infection of cells by bacteria, PBS or the materials at 500 μg/mL were supplemented and cultured for 3 h. Finally, paraformaldehyde solution (4%, v/v) was used to fasten the cells and then 4',6'-diamidino-2-phenylindole (DAPI) was used to stain the nucleus of NIH 3T3. At the excitation wavelength of 340 nm for DAPI and 490 nm for FITC, the situation of the antibacterial adhesion was observed by CLSM.

As for colony counting, the cells were washed three times with sterile PBS and lysed with Triton X-100 solution. Afterwards, the obtained samples were diluted in suitable

concentration, followed by being plated onto Luria−Bertani (LB) agar plates to determine the number of colonies.

10. Cytocompatibility Evaluation

NIH-3T3 cells were cultured in DMEM mentioned above at 37 °C in an incubator containing 5% CO_2. The cells were pre-cultured for 2-3 days before the experiment. First, the cells (100 μL, 5 × 10^4 cells mL^{-1}) were seeded into the 96-well plate and cultured for 24 h. Then the three kinds of nanobiocides (100 μL) with various concentrations (16, 32, 63, 125, 250, 500 μg/mL) and PBS were added in the wells and co-incubated with cells for another 24 h. After that, MTT working solution (10 μL, 0.5 mg/mL) was added, followed by incubating at 37 °C for 4 h. Next, the supernatant was discarded and DMSO (150 μL) was added into each group to dissolve the crystals under vibration for 15 min. Eventually, the absorbance at 570 nm evaluated by the microplate reader (Molecular Devices, USA) to calculate the cell viability:

$$\text{Cell viability} = (A_t - A_0)/(A_c - A_0) \times 100\% \quad (1)$$

which A_t represents the absorbance of treated cells, A_c represents the absorbance of the PBS group and A_0 represents the optical density of DMSO.

11. Hemolysis Analysis

To evaluate whether nanobiocides interacted with blood cells, hemolysis assay was conducted on human blood. The erythrocytes were collected via centrifugation at 1, 000 rpm for 3 min, washed three times with PBS solution, and finally diluted in saline to obtain an erythrocyte stock solution (5%). Then the stock dispersion and equal volume of the nanobiocide suspensions with various concentrations were incubated at 37 °C for

45 min. Besides, PBS and Triton X-100 that induced totally hemolysis were set as negative and positive control respectively. According to the following equation, the hemolytic percentage (hemolysis %) was determined by the absorbance of supernatant at 540 nm after centrifugation. In the equation, the absorption values of samples, PBS, and Triton-100 were denoted by A, Am, and An, respectively.

$$\text{Hemolysis } \% = (A-A_m)/(A_n-A_m) \times 100\% \quad (2)$$

12. Reactive Oxygen Species (ROS) Detection

2′,7′-Dichloro-fluorescein diacetate (DCFH-DA), an ROS trapping agent, was utilized to reflect the existence of ROS generated from samples under laser irradiation. Once combining with ROS, DCFH-DA could be converted into 2′,7′-dichloro-fluoresce (DCF) whose fluorescent emission is 525 nm. Nanobiocides with different degree of polymerizations and the blank control group were mixed with DCFH-DA (25 μg/mL) and illuminated with NIR laser (660 nm, 1 W/cm^2) for various time. Then the fluorescence intensity at 525 nm was measured qualitatively by the microplate reader (Tecan Freedom EVO200). Besides, fluorescence images of the samples were acquired under Caliper IVIS Lumina II.

13. Influence of illumination time on sterilization effect.

Aiming to explore the influence of illumination time on bactericidal effect concretely, the bacteria in the material group were illuminated for different time (0, 2, 4, 6, 8, and 10 min), and were quantified by standard plate counting assays. The concentration of nanobiocides were 250 μg/mL.

14. Detection of Bacterial Content.

Bacterial death is related to the destruction of membrane structure, and cellular contents (such as DNA and RNA) present strong absorption at 260 nm. We next measured the absorption value at 260 nm to determine the bacterial death. The experiment was divided into three groups: PBS (Dark), QDs@pG/F_{30} (Dark), QDs@pG/F_{30} (660 nm irradiation), and the absorption of suspension at 260 nm was measured after being filtered by percolators (220 nm).

15. Intracellular ROS Accumulation

The intracellular ROS accumulation experiment was performed according to the literature with slight modification.[4] Briefly, the nanobiocide was added into bacterial suspension to a final concentration of 500 μg/mL and incubated for 3 h. Then, 500 μL of DCFH-DA solution at 50 μg/mL was supplemented into the system, and cultured for another 15 min. The experiment was divided into six groups with different illumination times, 0, 2, 4, 6, 8 and 10 min, and PBS group without irradiating was set as the control. After being illuminated, all samples were rinsed twice with 0.85% saline, then re-suspended in 200 mL lysis buffer I (50 mM Glucose, 25 mM Tris-HCL, 10 mM EDTA) and followed by lysis buffer II (0.2 M NaOH, 1% SDS), after 3 min. The suspensions were centrifuged at 8 000 rpm for 10 min and the fluorescence was measured on a fluorescence spectrophotometer (E_x: 488 nm, E_m: 525 nm).

16. Live/Dead Staining Observation of Bacteria.

In order to visually observe the sterilization effect of the nanobiocide, the bacterial treated with QDs@pG/F_{30} and PBS were irradiated under 660 nm NIR for 10 min. After being washed three times with saline, *P. aeruginosa* were labeled with acridine orange

(AO) and ethidium bromide (EB), followed by several times of washing with PBS. Then the treated bacteria were suspended in a glycerol solution and observed under CLSM at appropriate excitation wavelength, 488 nm for AO and 518 nm for EB. This assay was carried out for each kind of *P. aeruginosa.*

17. Bacterial Morphology Observation

The morphologies of *P. aeruginosa* treated with various conditions were qualitatively estimated by SEM observation. Bacteria were divided into four groups: PBS (Dark), PBS (Illumination), QDs@pG/F_{30} (Dark) and QDs@pG/F_{30} (Illumination). The treated bacteria were washed with sterile PBS four times and dripped onto the silicon wafer, followed by fixing with 2.5% glutaraldehyde (J&K Chemical Reagent Co., China) solution for 4 h. Subsequently, the treated *P. aeruginosa* was dehydrated sequentially in different concentrations of ethanol solution (30%, 50%, 70%, 90% and 100%, v/v) for 10 min. Finally, the samples were imaged by SEM.

18. Standard Plate Counting Assays

To investigate the antibacterial efficiency of the material under laser quantificationally, bacteria treated with nanobiocides were calculated by standard colony counting method. Four different types of *P. aeruginosa* were detached into sterile PBS after centrifugation and divided into three groups: (I) PBS (Dark); (II) QDs@pG/F_{30} (Dark); (Ⅲ) QDs@pG/F_{30} (Iluumination). After 3 h of cocultivation, obtained suspension was diluted and planted onto LB agar plates and then incubated at 37 °C for 8 h. Afterwards, the colony-forming units (CFU) on different plates were imaged and counted.

19. Growth inhibitory assay to planktonic bacteria

Against planktonic *P. aeruginosa,* the growth inhibitory assay was assessed after being treated with materials. Concretely, *P. aeruginosa* cultured overnight was dispersed into LB medium by the concentration of OD_{600} = 0.2. The equal volume of therapeutics, with a series of concentrations (16, 32, 63, 125, 250 and 500 μg/mL) and PBS was mixed for 3 h of incubation, following illuminated for 10 min. Subsequently, the bacterial suspension was incubated for 8 h at 37 °C and the absorption at 600 nm was measured to determine the growth inhibition of bacteria. The IC_{50} value was introduced to measure the capacity to kill half of bacteria. The minimal inhibitory concentration (MIC) represents the minimum concentration to inhibit bacterial growth. Each sample was repeated in triplicate.

20. Twitching motility assay

Before researching the influence of materials on biofilm formation systematically, we preliminarily investigated the effect of glycomimetics on the twitching motility of bacterial flagellum.[5] After mixing three kinds of materials with semi-liquid LB agar, a certain amount of *P. aeruginosa* colony was stabbed into 1% LB agar plates till the bottom of the plate. After hatching at incubator for 48 h, the agar was removed and the bacterial motility zone was measured by staining with 1% crystal violet for 10 min. Then the excess dye was washed away, and twitching area diameters were quantified using Image J. Significant differences in twitching compared to the control group were determined by a one-way analysis of variance statistical test. Each material was tested in triplicate.

21. Inhibition of bacterial biofilm formation

Four strains of *P. aeruginosa*, PA14, PAK, PAO1 and MDR PA, were employed to evaluate the biofilm inhibition of nanotherpeutics. The bacterial suspension was added into the 96-well microliter plate at the concentration of OD_{600} = 0.05 and co-cultured with isometric 60 μL of materials at different concentrations (0, 16, 32, 63, 125, 250 and 500 μg/mL) at 37 °C for 24 h. After discarding planktonic bacteria with sterile PBS, we used methanol to fix the biofilms. Then, biofilm was stained with crystal and violet solution (0.5%, w/v) and finally each wall was filled with 33% acetic acid to release the dye. Ultimately, the absorbance at 590 nm of each well determined the effect of biofilm formation and each sample was repeated three times. We used BIC_{50} to evaluate the lowest concentration of biofilm formation at 50% reduction. To intuitively observe the inhibition effect of materials, the biofilm was stained by using FITC-labeled Concanavalin A (ConA-FITC) and EB, and imaged with three-dimensional (3D) CLSM.

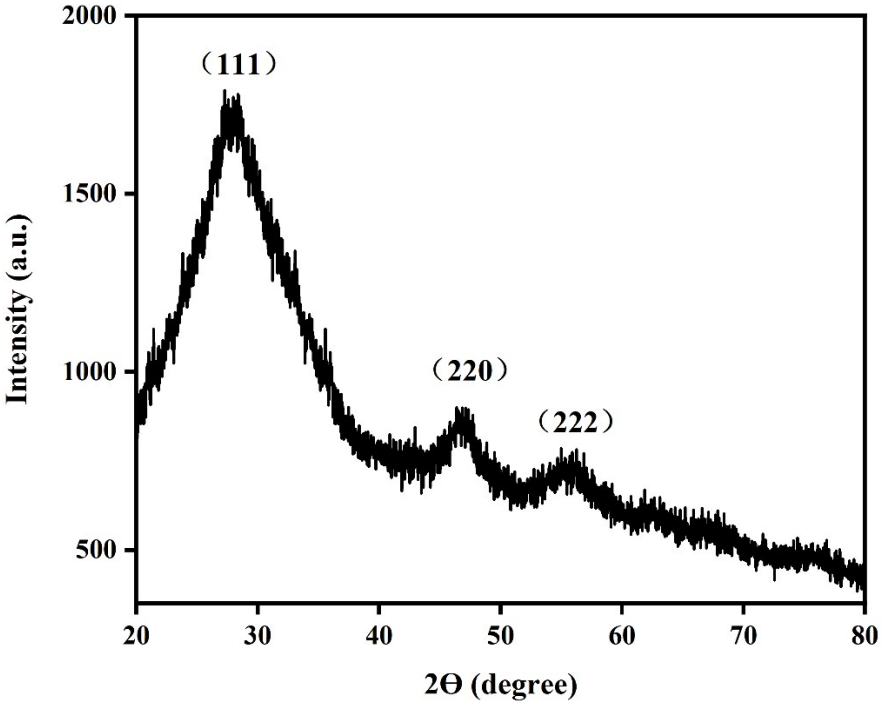

Fig. S1 X-ray diffraction patterns of ZCIS QDs

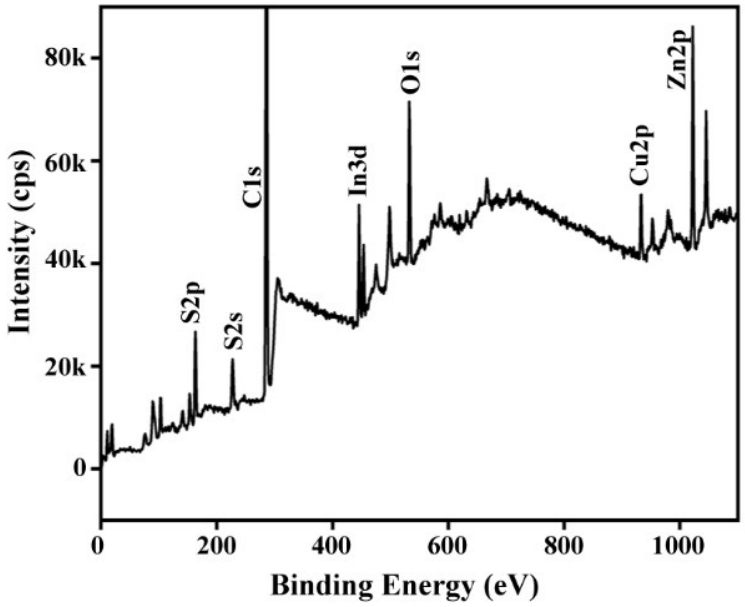

Fig. S2 XPS survey spectrum of ZCIS QDs containing Cu, In, S and Zn.

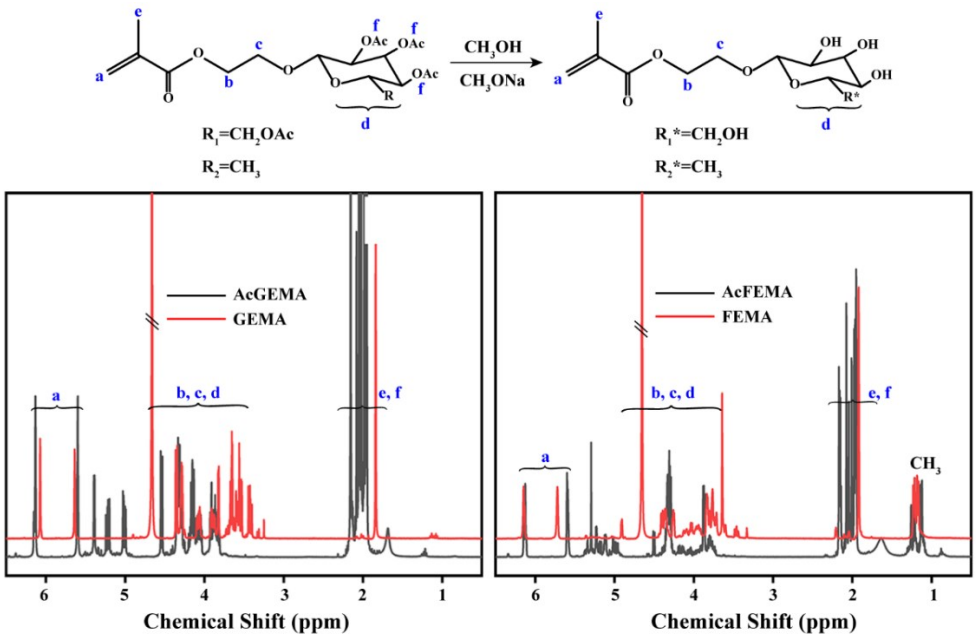

Fig. S3 Synthsis of deprotection of AcGEMA and AcFEMA, ^{1}H NMR spectra of AcGEMA and AcFEMA in $CDCl_3$, GEMA and FEMA in D_2O.

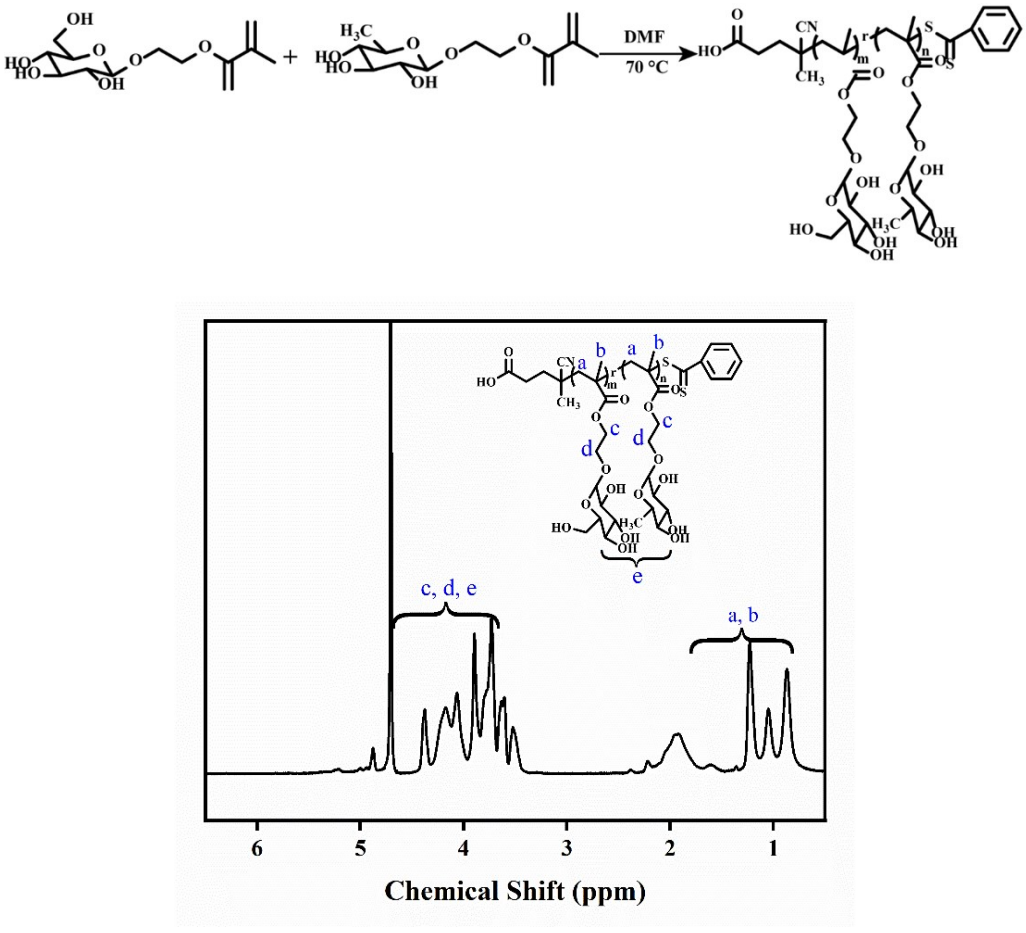

Fig. S4 Synthsis and ^{1}H NMR spectra of p(GEMA-*r*-FEMA).

Table S1. Constitution of Copolymers

Sample	Conv[a] (wt %)	*Mn*[b] (kDa)	*Mn*[c] (kDa)	PDI
p($FEMA_{10}$-*r*-$GEMA_{10}$)	70	11.3	8.3	1.37
p($FEMA_{20}$-*r*-$GEMA_{20}$)	75	12.6	9.1	1.25
p($FEMA_{30}$-*r*-$GEMA_{30}$)	63	15.0	10.4	1.44

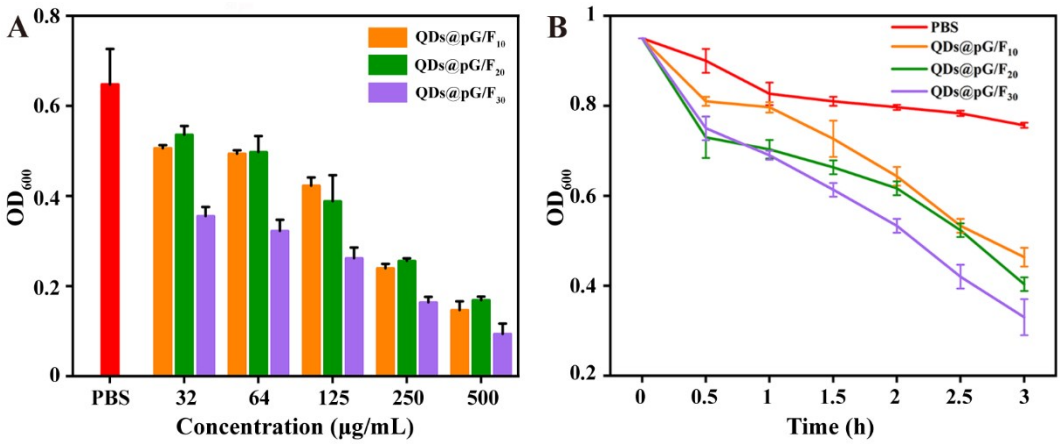

Fig. S5 Adhesion of nanobiocides toward *P. aeruginosa.* The OD_{600} value of the supernatant as a function of (A) concentration, and (B) adhesion time after the incubation of $QDs@pG/F_{10,}$ $QDs@pG/F_{20}$ and $QDs@pG/F_{30}$ with *P. aeruginosa*.

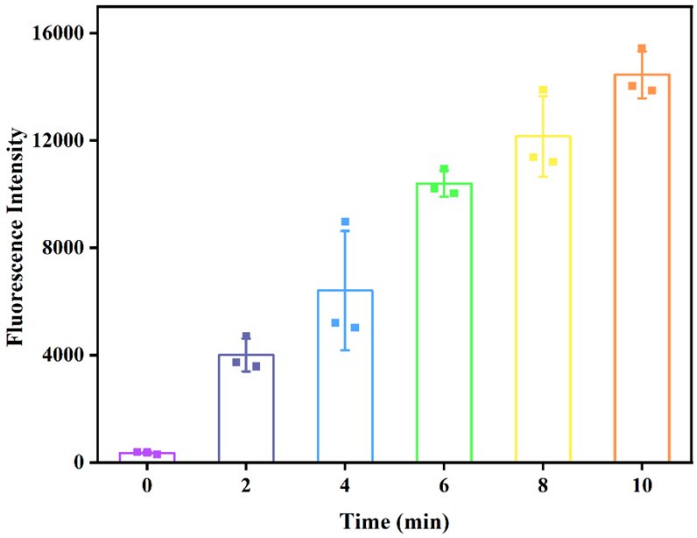

Fig. S6 The intracellular ROS accumulation under different illumination time.

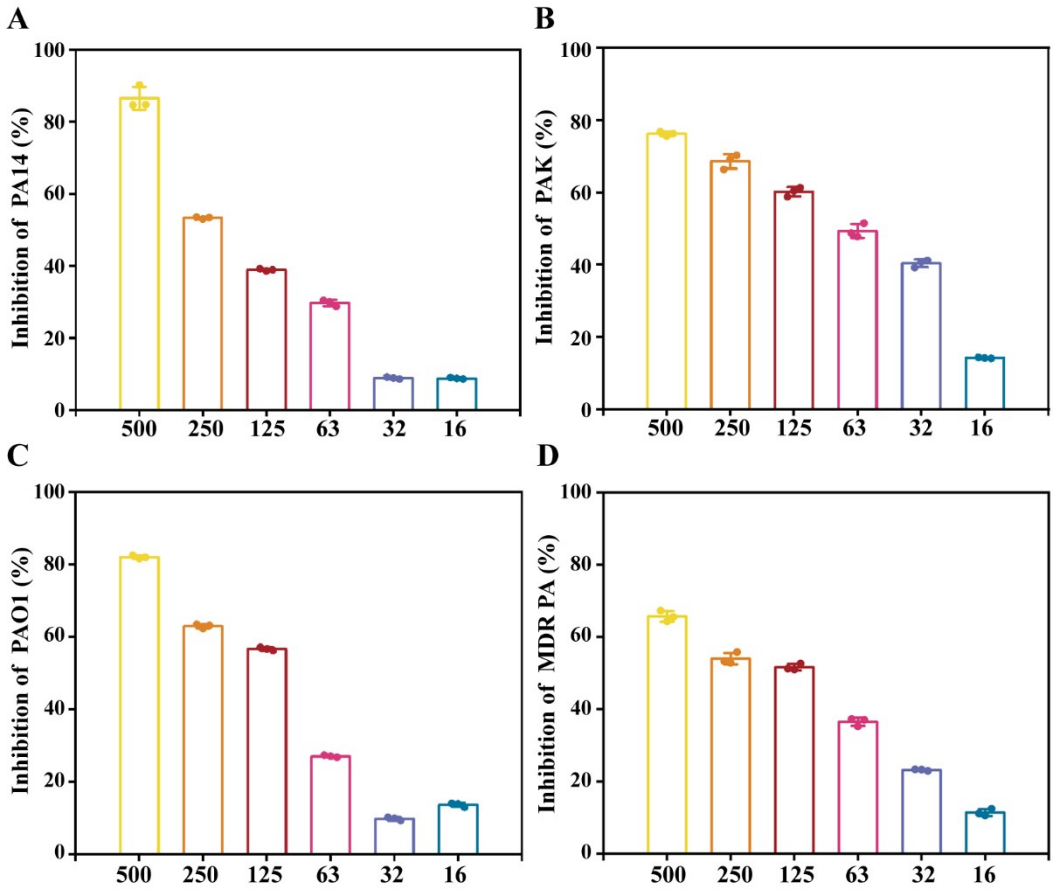

Fig. S7 Growth inhibition of QDs@pG/F_{30} to four strains of *P. aeruginosa*, (A) PA14, (B) PAK, (C) PAO1, and (D) MDR PA.

References

1. Cheng, C.; Zhang, X.; Wang, Y.; Sun, L.; Li, C., Phenylboronic acid-containing block copolymers: synthesis, self-assembly, and application for intracellular delivery of proteins. *New J. Chem.* **2012,** *36* (6), 1413-1421.

2. Guo, W.; Chen, N.; Tu, Y.; Dong, C.; Zhang, B.; Hu, C.; Chang, J., Synthesis of Zn-Cu-In-S/ZnS core/shell quantum dots with inhibited blue-shift photoluminescence and applications for tumor targeted bioimaging. *Theranostics* **2013,** *3* (2), 99-108.

3. Dai, X.; Bai, Y.; Zhang, Y.; Ma, Z.; Li, J.; Sun, H.; Zhang, X., Protonation–Activity Relationship of Bioinspired Ionizable Glycomimetics for the Growth Inhibition of Bacteria. *ACS Applied Bio Materials* **2020,** *3* (6), 3868-3879.

4. Xiao, Q.; Mai, B.; Nie, Y.; Yuan, C.; Xiang, M.; Shi, Z.; Wu, J.; Leung, W.; Xu, C.; Yao, S. Q.; Wang, P.; Gao, L., In Vitro and In Vivo Demonstration of Ultraefficient and Broad-Spectrum Antibacterial Agents for Photodynamic Antibacterial Chemotherapy. *ACS Appl Mater*

Interfaces **2021**.

5. Kilmury, S. L. N.; Burrows, L. L., The Pseudomonas aeruginosa PilSR Two-Component System Regulates Both Twitching and Swimming Motilities. *mBio* **2018,** *9* (4).