Supporting Information

Low-toxicity carbon quantum dots derived from gentamicin sulphate to combat antibiotic resistance and eradicate mature biofilms

Experimental section

Materials and Instruments

Gentamicin sulfate were obtained from Heowns Biochemical Technology Co., Ltd. (Tianjin, China). Drug-sensitive bacterial strains, *Staphylococcus aureus* (*S. aureus*, ATCC 25923) and *Escherichia coli* (*E. coli*, ATCC 25922), were purchased from Nanjing Lezhen Biotechnology Co., LTD (Nanjing, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), LIVE/DEAD viability/cytotoxicity kit, penicillin-streptomycin and other cell cultural media were obtained from Gibco BRL (Grand Island, NY, USA). The Luria broth (LB) media and tryptic soy broth (TSB) media were come from Aladdin (Shanghai, China). 2', 7'-dichlorofluorescin-diacetate (DCFH-DA) was received from Jiancheng Bioengineering Institute (Nanjing, China).

Fourier transform infrared (FTIR) spectra were carried out with Nicolet iS10 (Thermo Fisher Scientific, USA). UV−vis absorption spectra were carried out by a Biomate 3S spectrophotometer (Thermo Fisher Scientific, USA). Fluorescence emission spectrums of CQDs were read with an F-2700 fluorescence (FL) spectrophotometer (Hitachi, Japan). High-resolution transmission electron microscopy (HRTEM) images was obtained by a

JEM-2100 electron microscope at 200 kV (JEOL Ltd, Japan). X-ray photoelectron spectroscopy (XPS) was evaluated by a VG ESCALAB MKII spectrometer. High performance liquid chromatography combined with mass spectrometry (HPLC-MS) of gentamicin sulphate was performed on a Waters 2996/Waters micromass ZQ. The mobile phase was acetonitrile/water with the volume ratio of 0.05 %. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was conducted on an UltrafleXtreme MALDI-TOF mass spectrometer (Bremen, Germany). Thermogravimetry (TG) combined with differential scanning calorimetry (DSC) were carried out from 25 ℃ to 800 ℃ using a Mettler Tory analyzer at a heating rate of 10 ℃/min with a nitrogen flow of 100 cm^3/min. Biofilm images were performed on a field emission scanning electron Microscope (FESEM) TSM-7500F (JEOL Ltd. Japan) at 10.0 kV. Fluorescence photograph of confocal laser scanning microscope (CLSM) was investigated to observe the viability and death of bacteria in biofilm.

Synthesis of CQDs from Gentamicin sulfate (CQD_{Gents})

Firstly, Gentamicin sulfate was dissolved in 5 mL deionized water by ultrasound in crucible. The crucible with sample was then placed in a muffle furnace, heated to a certain temperature (150, 160, 170, 180, 190, 200, 210, 220, 230, 240 and 250 ℃) within 30 minutes and calcined for 2 hours at that temperature, and then cooled naturally. Then 10 mL deionized water was added to the crucible and dissolved by ultrasound for 1 hour. The turbid liquid was centrifuged for 30 minutes at 15000 r/min, and the supernatant was dialyzed in 3000 Da dialysis membrane for 48 hours. The prepared CQDs solutions were

then lyophilized to yield powders, stored at 4 ℃.

Detection of Minimum Inhibitory Concentration (MIC)[1]

Before the MIC experiment, the bacteria on the slope of solid medium were cultured in liquid Luria Broth (LB) medium for 18 hours, including *S. aureus* and *E. coli*. In the sterilized 96-well plate, 100 μL LB medium was added to each well, and a certain concentration of CQDs was added to the first well and a serial two-fold dilutions were made. Then 80 μL liquid medium was added to each well, and 20 μL bacterial suspension with concentration of 2×10^7 CFU/mL was added to wells at last. The MICs were determined by turbidity method, that is, the concentration of CQDs corresponding to the well without turbidity was the MIC value.

Inhibition zone test[2]

First, 10 mm filter papers were immersed in the materials (1 mg/mL) for 10 minutes. Then, *S. aureus* or *E. coli* were coated on LB solid medium. Finally, each soaked filter paper was attached to the middle of the culture plate. The plates were placed in a constant temperature and humidity incubator at 37 ℃ for 24 hours.

Evaluation of bacterial resistance to CQDs[3]

The initial MIC values of control antibiotics Gentamicin and different CQDs against *S. aureus* and *E. coli* were obtained as described above. Bacteria from 96-well plate at the concentration of one-half MIC were then used to prepare the bacterial dilution (about 10^7 CFU/mL) for the next MIC detection. These bacterial suspensions were then incubated with control antibiotics Gentamicin and different CQDs respectively. The new MIC values

were obtained after incubation at 37°C for 24 h. The same experiment was repeated every day for 28 passages.

Observation of Bacterial Cell Membrane Destruction by Field Emission Scanning Electron Microscope (FESEM)[4]

Planktonic bacteria (1.25×10^7 CFU/mL for *S. aureus* and 2.0×10^8 CFU/mL for *E. coli*) and CQD_{180} (6 μg/mL) were mixed in 6-well plate and cultured in table concentrator at 37 ℃ for 1 hour. After incubation, 2 mL 2.5% glutaraldehyde was used to immobilize the morphology of bacterial cells. The redundant glutaraldehyde was removed and the treated bacteria were washed with PBS for three times, followed by gradient dehydration of fixed bacteria with 30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol. Finally, the morphology of bacterial cells was observed under scanning electron microscopy after drying at the critical point of carbon dioxide and spraying gold.

Intracellular ROS detection of *S. aureus* stimulated by CQD_{Gents}

Since the non-fluorescent fluorescent 2′,7′-Dichlorodihydrofluorescein Diacetate (DCFH-DA) can be decomposed by the cellular esterase into a non-fluorescent 2-(2,7-Dichloro-3,6-dihydroxy-9H-xanthen-9-yl) benzoic acid, which would be oxidized by the reactive oxygen species to fluorescent 2', 7'-dichlorodihydrofluorescein (DCF)[5]. The DCF has a fluorescence emission peak centered at 525 nm when excited at 488 nm. In short, the *S. aureus* suspension was added to each sterile vial and treated with 2 mL DCFH-DA (10 μM) aqueous or phosphate buffered solution (pH 7.4, control), respectively. Subsequently, gentamicin (80 μg/mL), CQD_{Gents} (80 μg/mL), a positive control (1 mM hydrogen

peroxide) or a negative control (phosphate buffered solution, pH 7.4) were added to the vials, respectively. Finally, the fluorescence intensity was observed with a fluorescence spectrophotometer having an excitation wavelength of 488 nm. The content of reactive oxygen species is proportional to the fluorescence intensity of DCF.

Destruction of mature *S. aureus* biofilm by CQDs

First, fresh bacterial suspension was prepared by shaking *S. aureus* in LB medium for 18 hours. 200 μL Tryptone Soybean Broth (TSB) medium was added to the 96-well sterile plate, and 10 μL 10^5 CFU/mL bacteria were inoculated into each well. Then 96-well plates were placed in a constant temperature and humidity incubator for 24 hours. *S. aureus* proliferate continuously during cultivation and form compact white biofilm at the bottom of the wells. Next, the culture media were gently removed and the biofilm was washed with PBS for three times. Then 200 μL CQDs TSB solutions were added to each well, and the static culture was continued in the incubator for another 24 hours. Then, the supernatant planktonic bacteria and CQDs were removed and washed with PBS for three times. In this paper, there were three methods to detect the destruction of biofilm by CQDs. The first method was the crystal violet staining experiment.[6] The destroyed biofilm was dried at 60 ℃ in an oven. Then 200 μL methanol was added to immobilize the biofilm for 15 minutes. After immobilization, methanol was removed and dried at 60 ℃, 100 μL 0.1% crystal violet was added to stain the nucleic acid of alive *S. aureus* in biofilm. The staining time was 15 minutes. Then, the redundant crystal violet was removed and washed by PBS for three times. After drying at 60 ℃, the biofilm was decolorized with 200 μL 95% ethanol. The

process of decolorization was carried out in table concentrator with time of 30 min. Finally, the absorption value of the mixed solution at 570 nm was measured by a micro plate reader, and the destructive rate of each CQDs to the biofilm was calculated by following formula. The second method is to observe the morphology of the damaged biofilm by scanning electron microscopy (SEM).[7] The experimental process was as follows. The biofilm destroyed by CQDs was immobilized by 2.5% glutaraldehyde for 24 hours, then washed with PBS three times, and gradient elution of the immobilized biofilm was carried out with 30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol solution. After drying at the critical point of carbon dioxide and spraying gold, the morphology of biofilm was observed. The third method was to observe the state of biofilm after destruction by confocal laser scanning microscope (CLSM).[8] AO/EB were used to stain biofilm. Green light represented living bacteria and red light represented dead bacteria. The ability of CQDs to destroy biofilm was evaluated by observing the live and dead state of bacteria in biofilm.

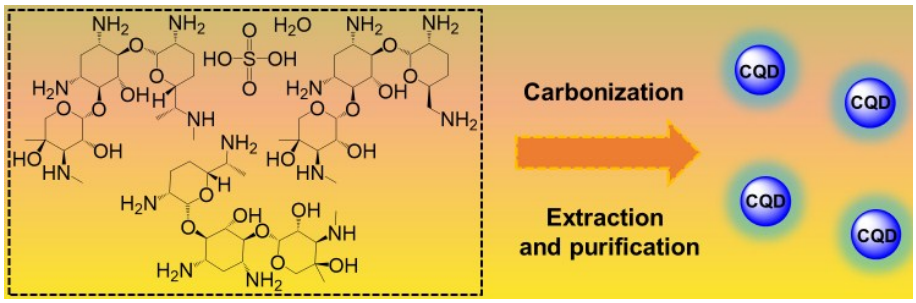
$$\text{Destruction ratio}\ (\%) = \frac{OD_{570nm}{}^{(Control)} - OD_{570nm}{}^{(sample)}}{OD_{570nm}{}^{(Control)}} \times 100\%$$

***In vitro* Cytotoxicity test**[9]

The *in vitro* cytotoxicity of the CQDs to mammalian cells was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The HEK 293T, HUVEC, L929 and 3T3 were selected as the experimental cells. Firstly, model cells (1×10^5 cells per well) were seeded in T25 flask and cultured for 24 h. The condition of culture was in Dulbecco's modified Eagle's medium (DMEM), which containing 10% fetal

bovine serum (FBS) and 1% penicillin-streptomycin. The cells were incubated at 37 °C with 5% CO_2 in incubator. Next, different concentrations of CQDs solution were placed in 96-well plates. And the untreated group was served as control. Subsequently 200 μL of DMEM containing 5×10^3 cells were added to each well. Then the samples were incubated for 24 hours in a 5% CO_2 incubator. And then the DMEM in each well was removed. The wells were washed with PBS for three times. 10 μL of the MTT solution (5 mg/mL) was added to each well. Soon afterwards, the cells were incubated in incubator for another 4 hours. During cultivation, the reaction of MTT and succinate dehydrogenase in mitochondrion happened to form blue formazan when dissolved in dimethyl sulfoxide (DMSO, 200 μL). Lastly, the absorbance of blue formazan was detected using a micro plate reader at 540 nm. This experiment was repeated for three times. Cell viability ratio of control group was deemed as 100 %. The relative cell survivals (%) after treated with CQDs at different concentrations were calculated using the following formula:

$$\text{Relative cell viability}\,(\%) = \frac{\text{OD}_{540\text{nm}}(\text{Sample})}{\text{OD}_{540\text{nm}}(\text{Control})} \times 100\%$$



Scheme S1 Synthesis route of CQD_{Gents}.

Table S1 Yield of CQD_{Gents}

Temperature (℃)	150	160	170	180	190	200	210	220	230	240	250
Yield rate (%)	8.9	13.2	21.3	24.6	26.1	27.9	28.7	29.2	20.1	13.4	10.2

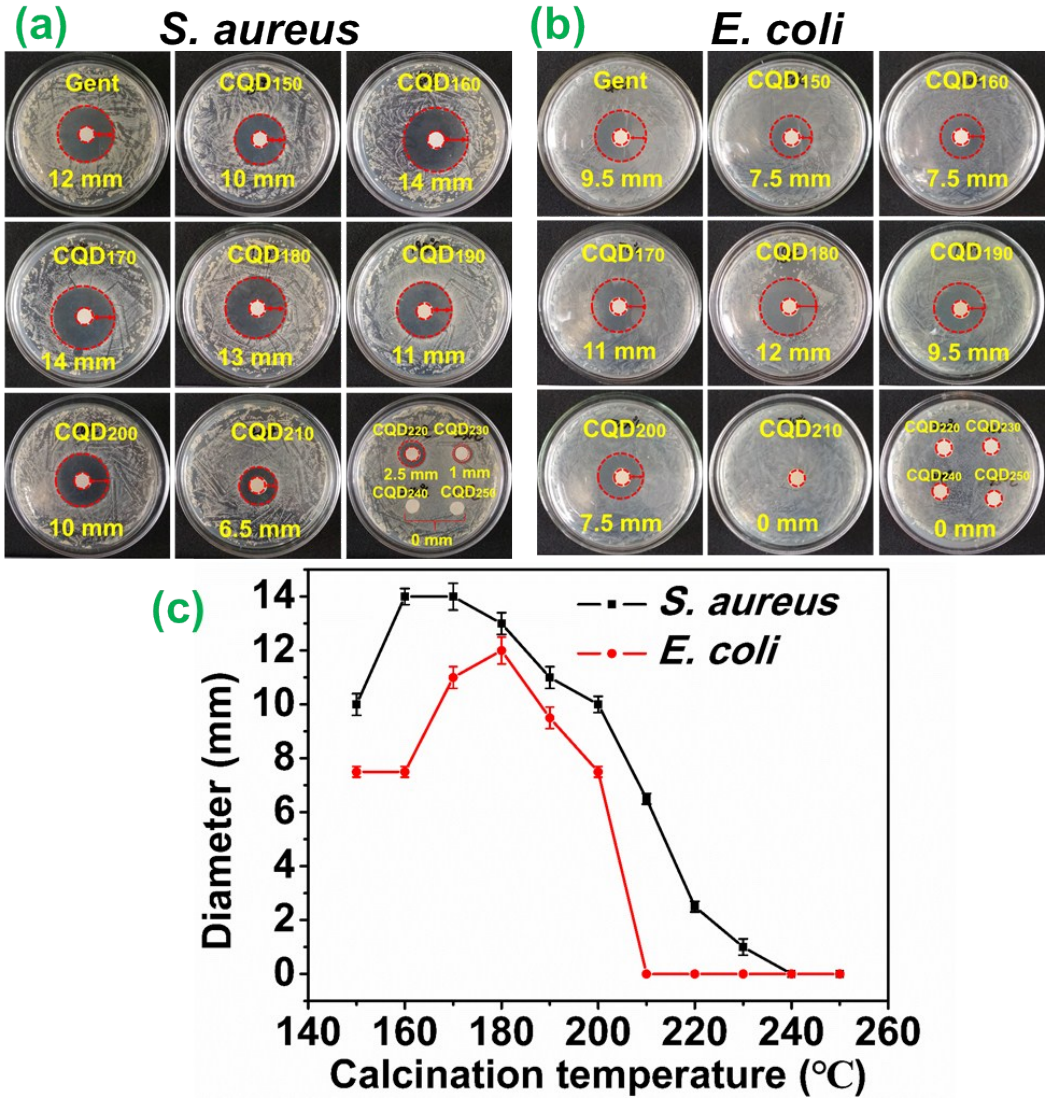


Fig. S1 Inhibition zone of CQD_{Gents} (1 mg/mL) against (a) *S. aureus* and (b) *E. coli*. (c) Curves of inhibition zone for different CQD_{Gents} against *S. aureus* and *E. coli*.

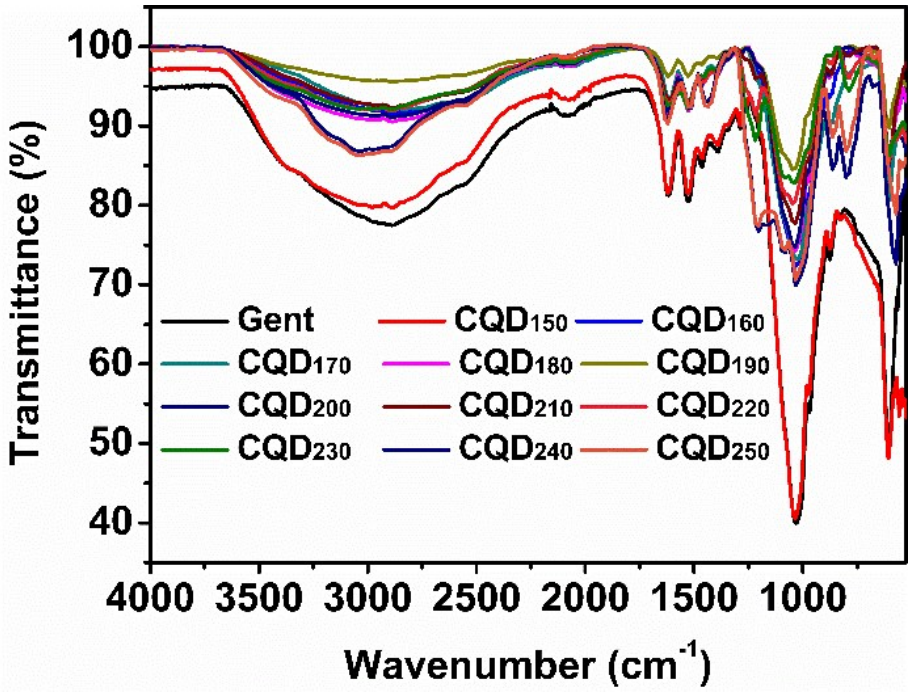


Fig. S2 Fourier Transform Infrared (FTIR) spectroscopy of gentamicin and CQD_{Gents}.

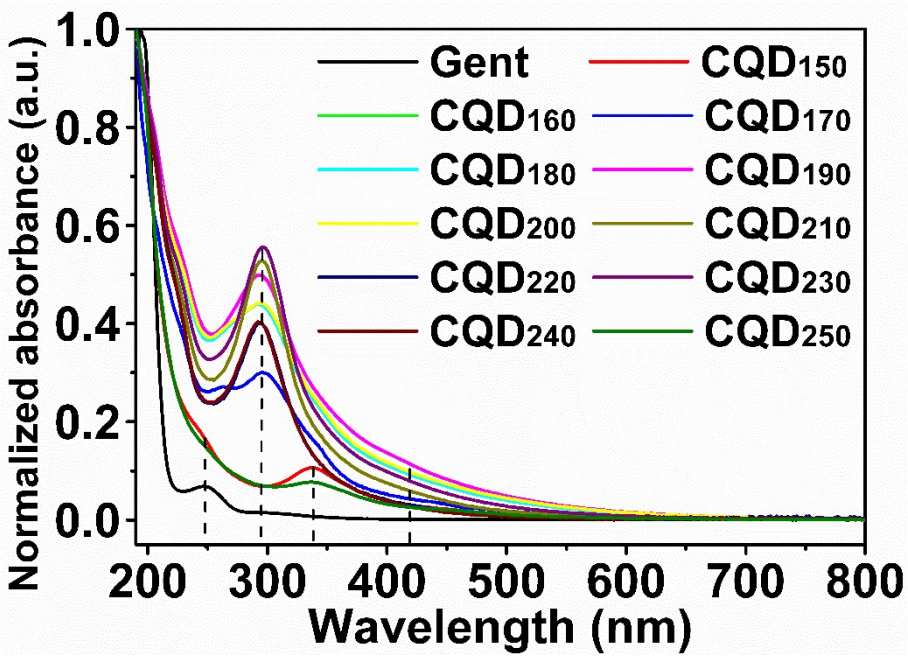


Fig. S3 UV-Vis spectrum of CQD_{Gents} calcined at different tempretures.

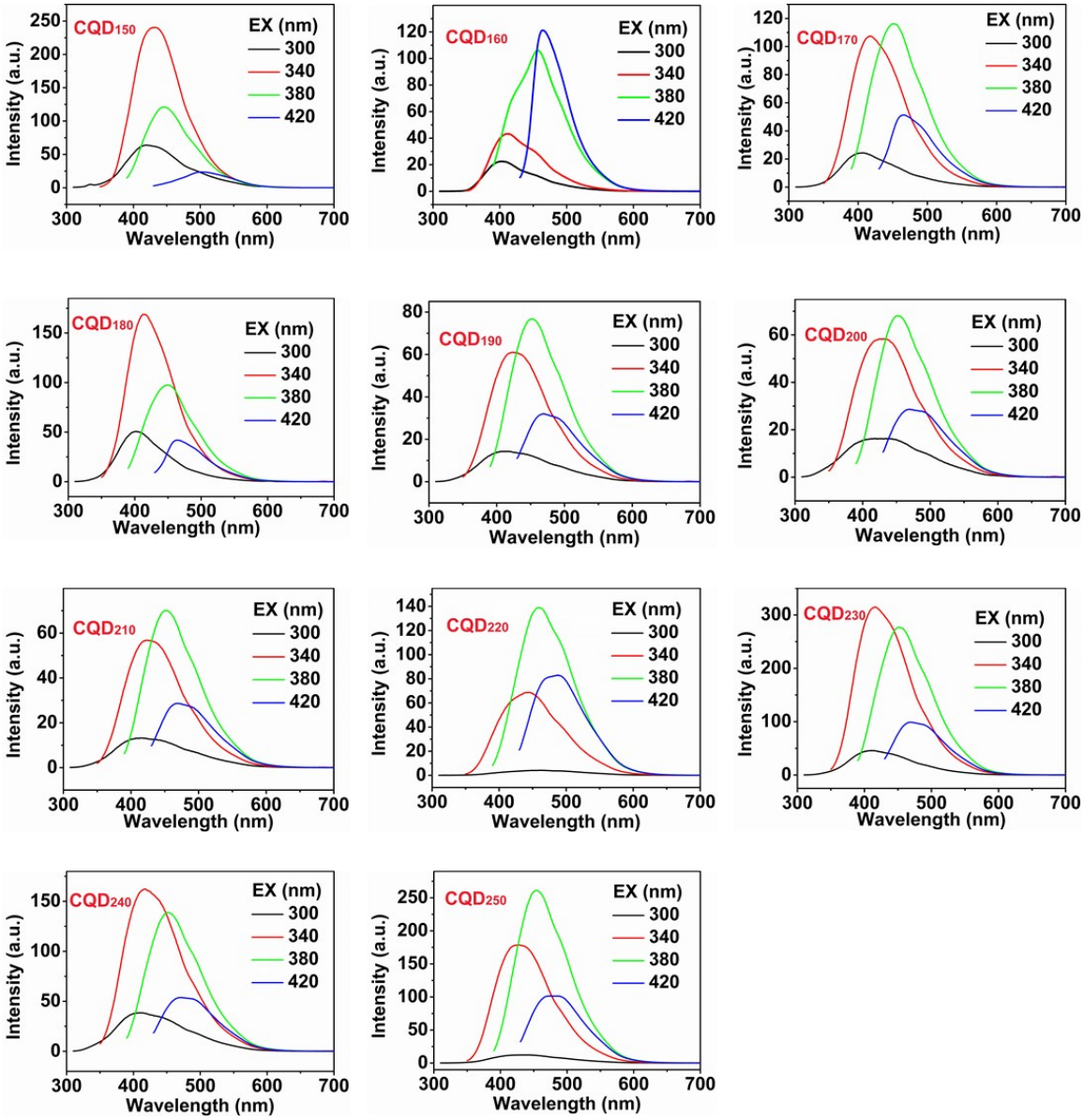


Fig. S4 Fluorescent spectrums of different CQD_{Gents} using a fluorescence spectrophotometer.

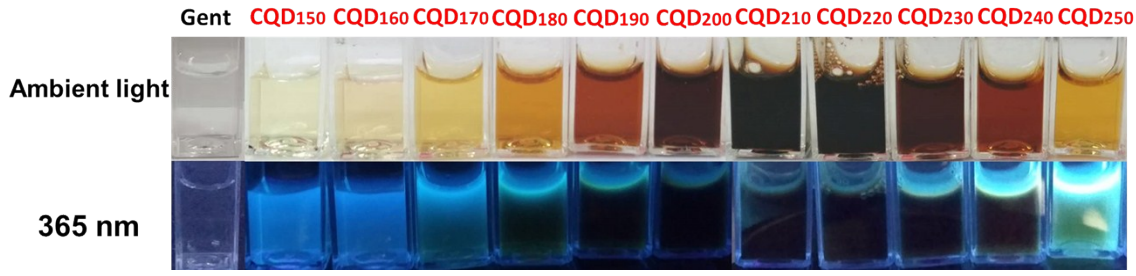


Fig. S5 Optical pictures of CQD_{Gents} irradiated at ambient and 365nm light respectively.

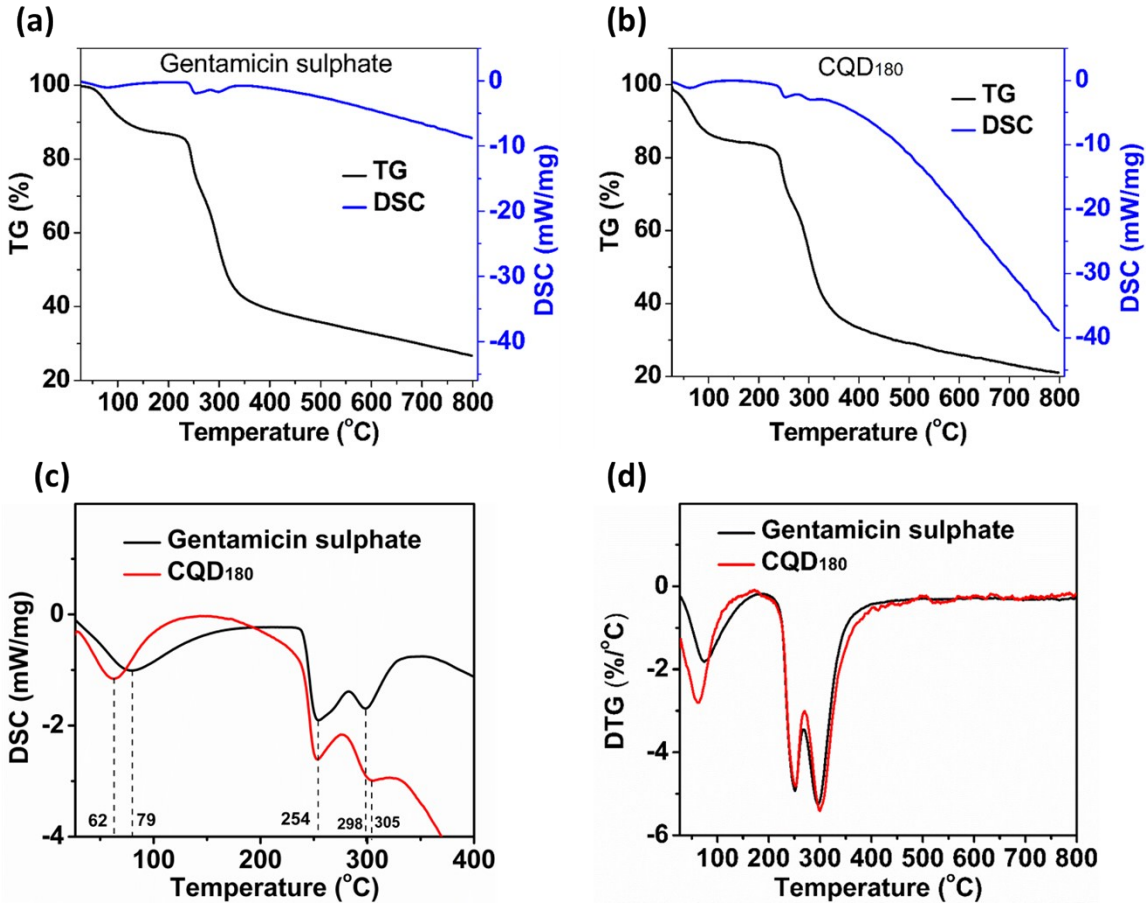


Fig. S6 Thermogravimetry (TG) and differential scanning calorimetry (DSC) curves for (a) gentamicin sulphate and (b) CQD_{180} in a dynamic nitrogen atmosphere, respectively, at 30 mL/min at a heating rate of 10 ºC/min. (c) Enlarged view of DSC curves. (d) Derivative TG profile of gentamicin sulphate and CQD_{180}.

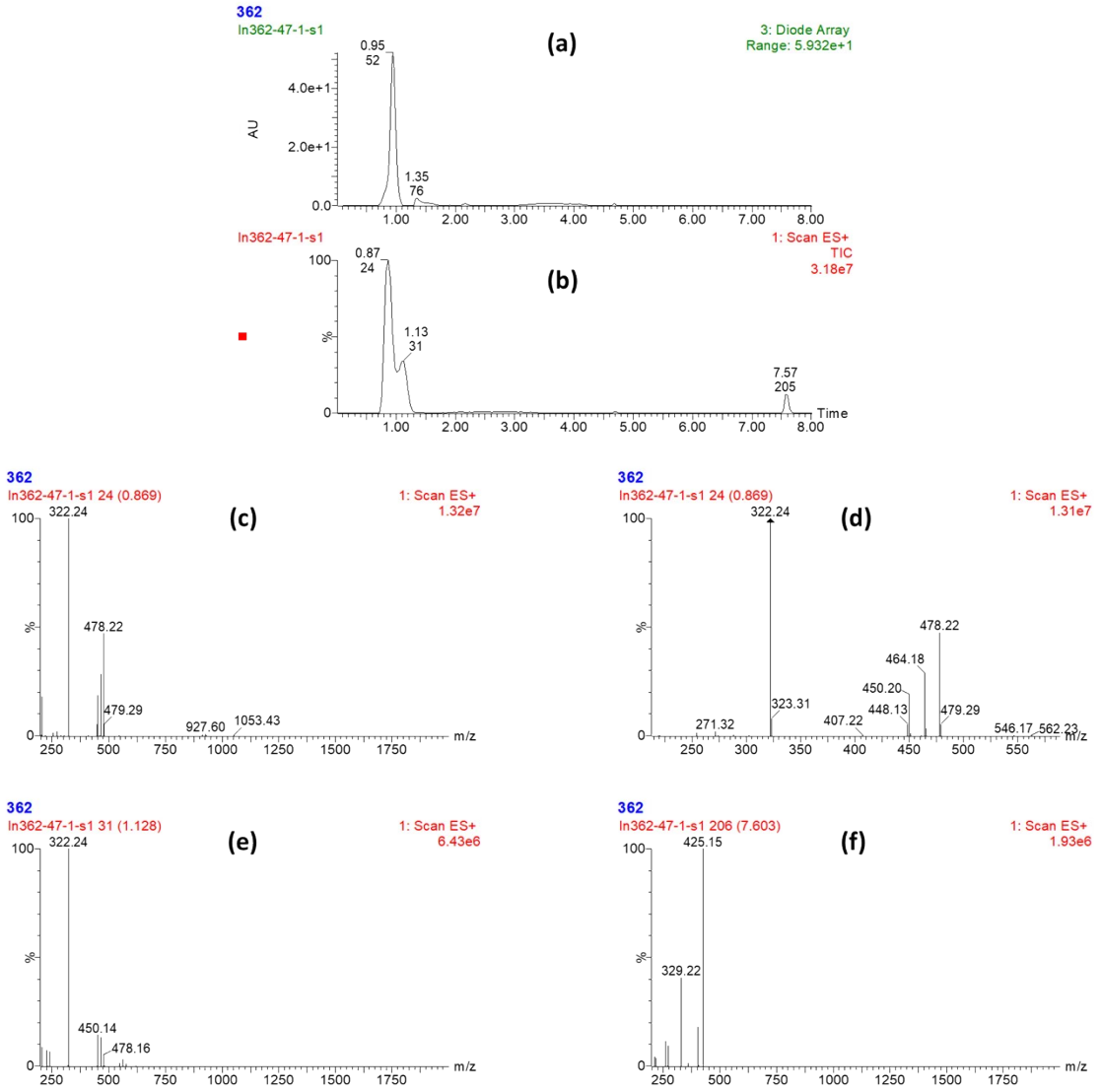


Fig. S7 High performance liquid chromatography combined with mass spectrometry (HPLC-MS) of gentamicin sulphate. (a) Liquid chromatography of gentamicin sulphate. (b) Ion current chromatograms of gentamicin sulphate of different retention time. (c-f) Mass spectra of gentamicin sulphate of different retention time.

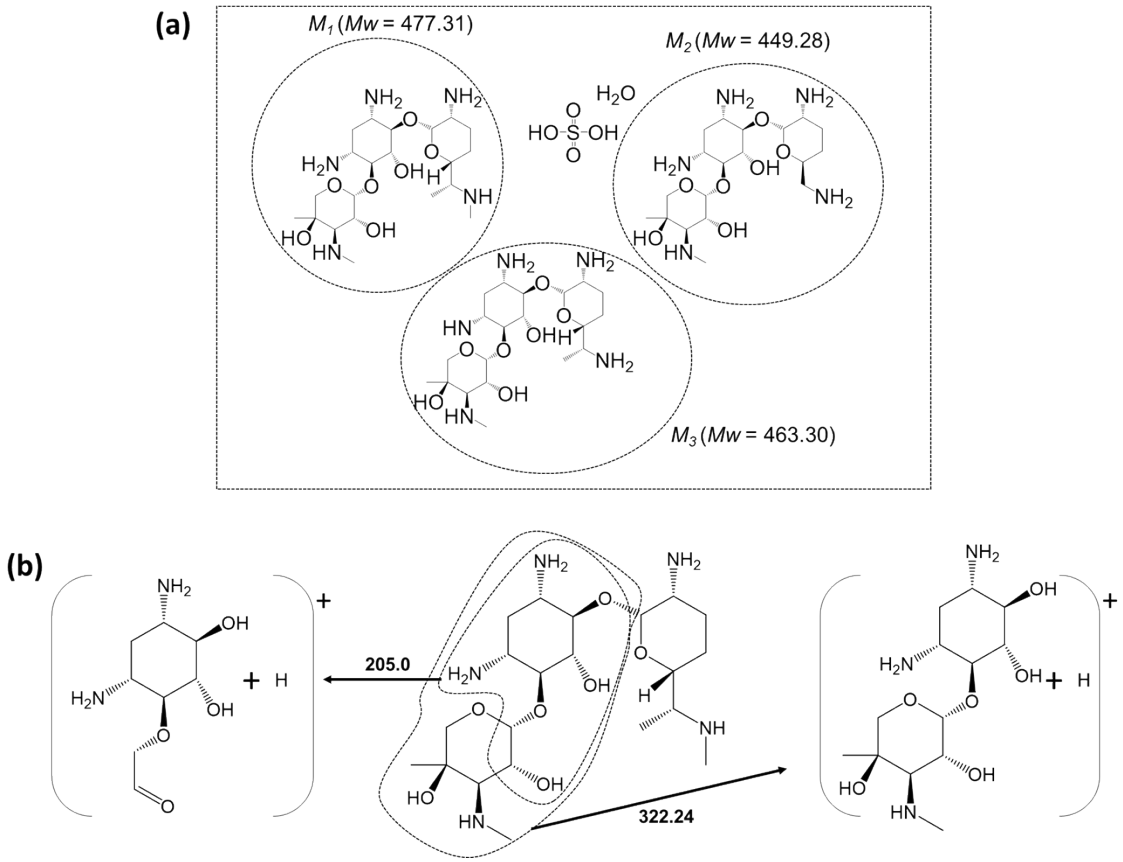


Fig. S8 (a) Molecular structure of gentamicin sulphate and the molecular weights of the three part. (b) Fragmentation scheme proposed for the gentamicin products for fragment ions at m/z 205.0 and 322.24.

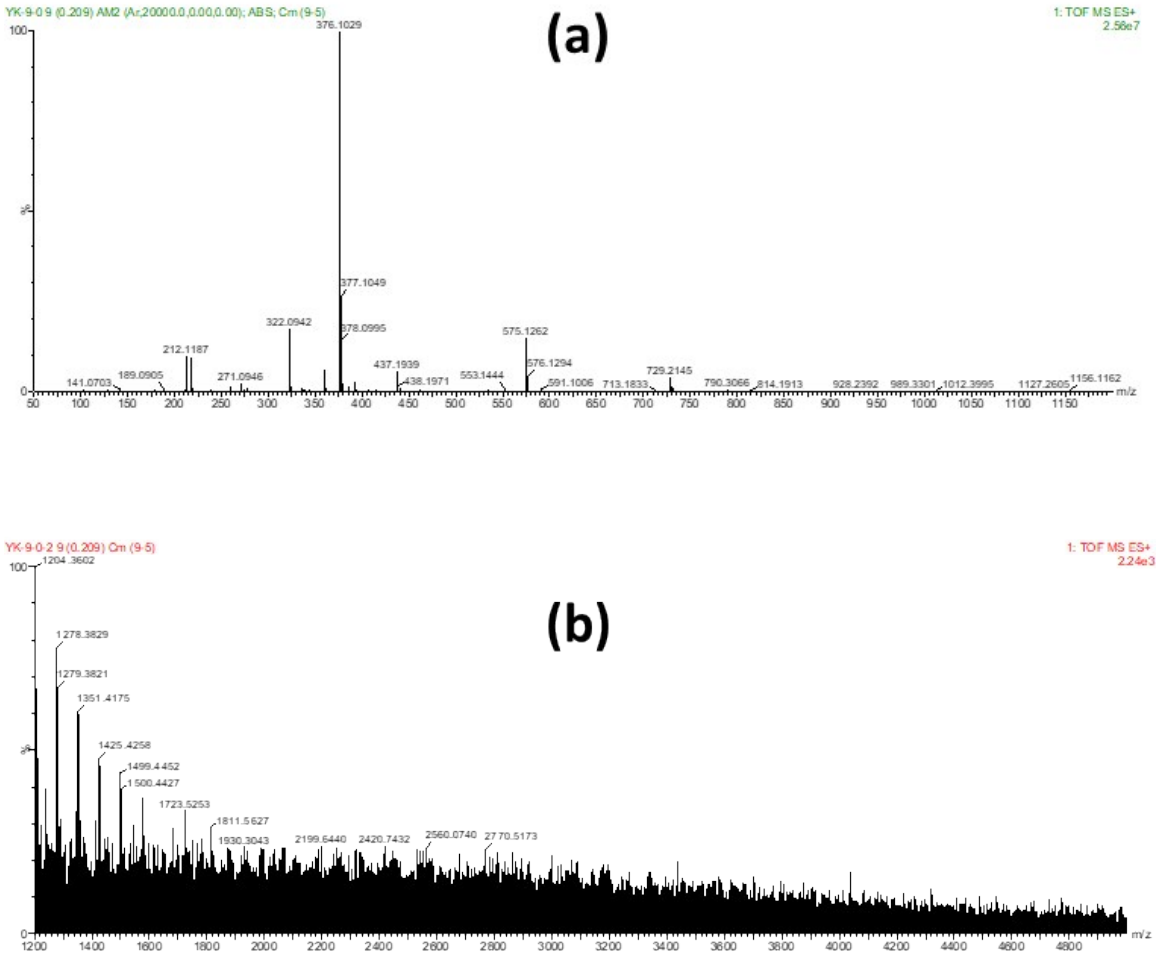


Fig. S9 MADLI-TOF mass spectrum of CQD_{180}. (a) The range of m/z is from 50 to 1200. (b) The range of m/z is from 1200 to 5000.

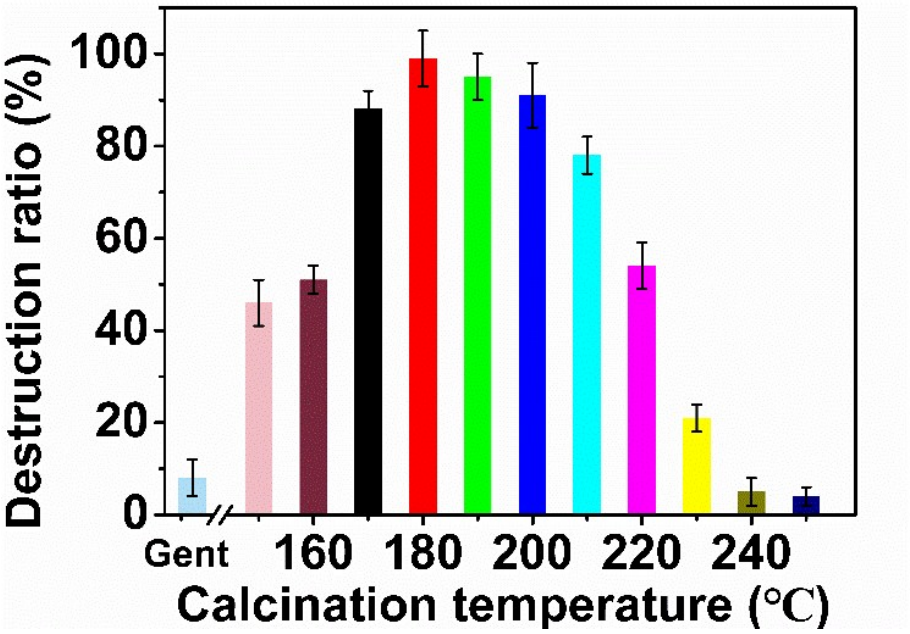


Fig. S10 Destruction ratio of CQD_{Gents} for *S. aureus* biofilm. (80 μg/mL)

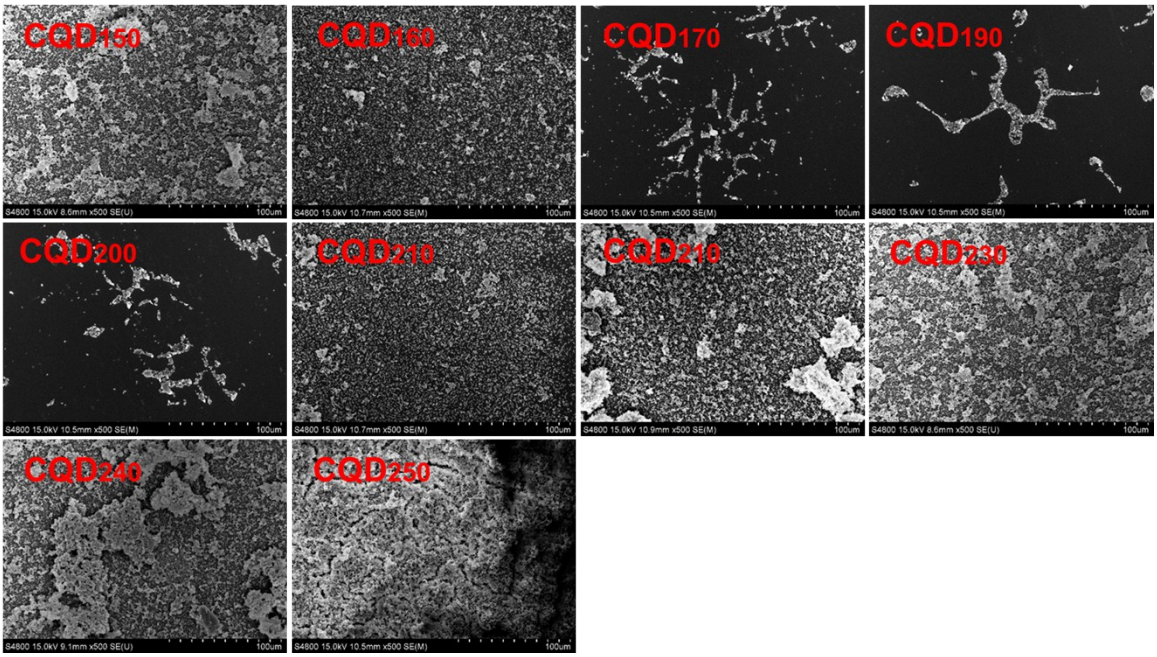


Fig. S11 Scanning electron microscopy (SEM) images of mature *S. aureus* biofilm treated with gentamicin (80 μg/mL) and CQD_{Gents} (80 μg/mL).

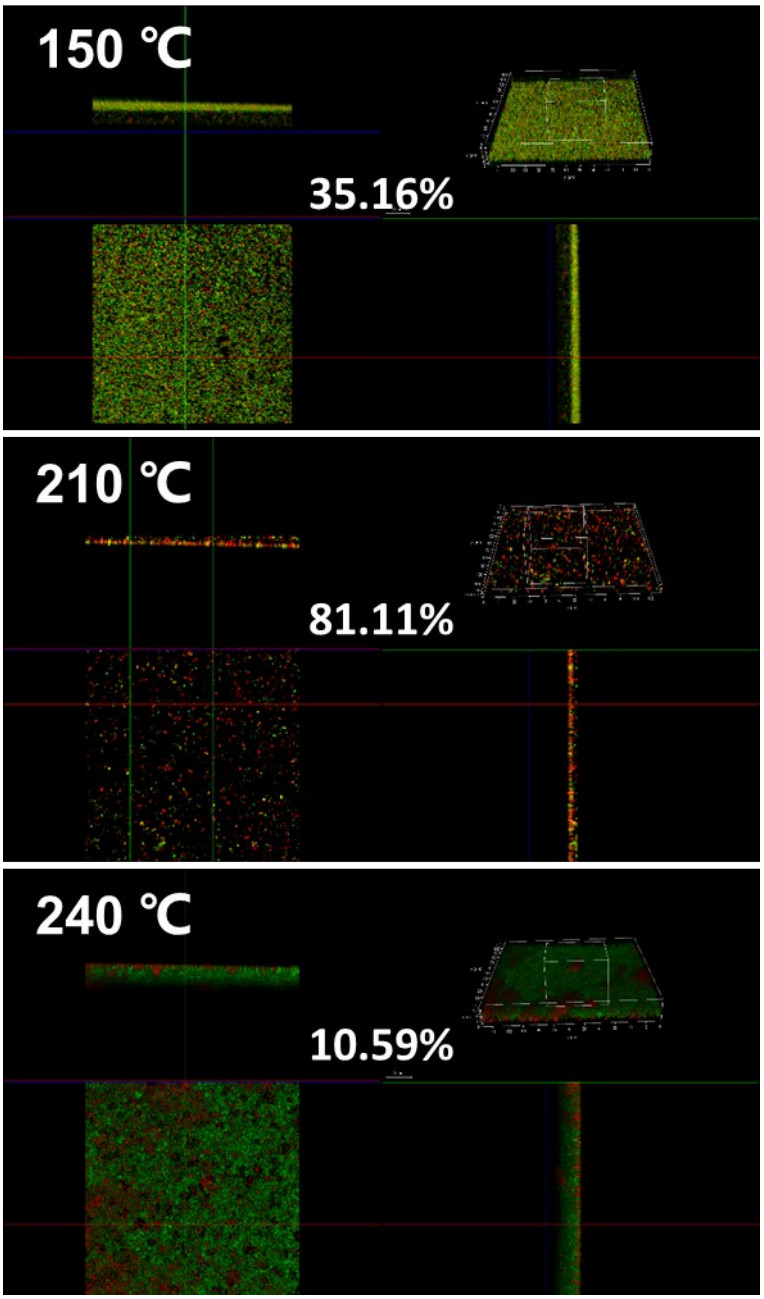


Fig. S12 Confocal laser scanning microscope (CLSM) images of mature *S. aureus* biofilm treated with gentamicin (80 μg/mL), CQD_{150}, CQD_{210} and CQD_{240} (80 μg/mL). The percent in the images are biofilm destruction ratio.

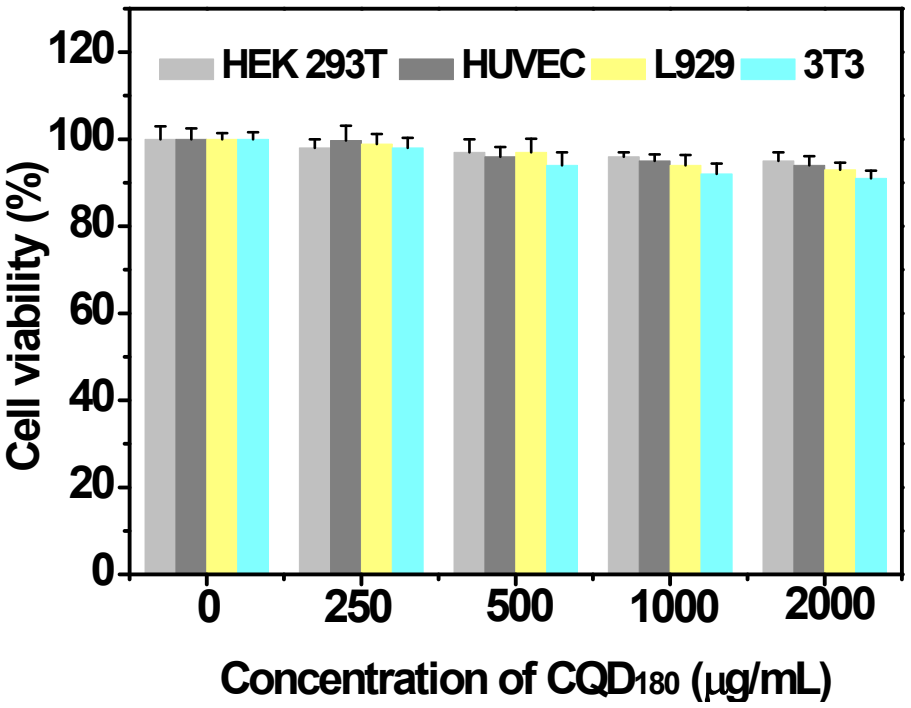


Fig. S13 Toxicity of CQDs to a panel of mammal cells.

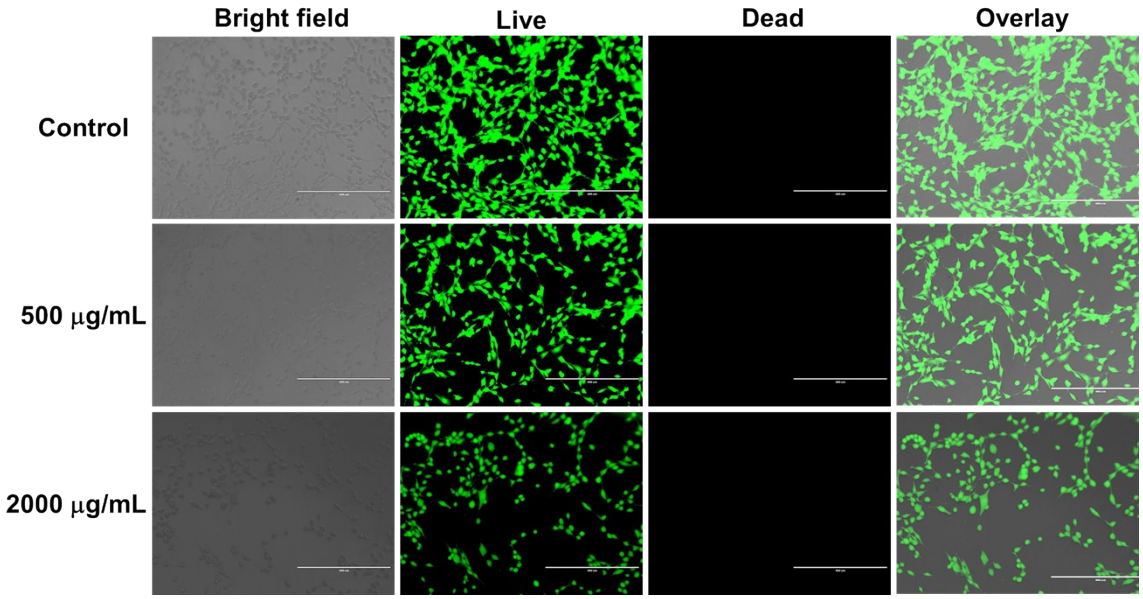


Fig. S14 Fluorescence images of 3T3 cells after the treatment with CQD_{180} for 24 h.

References

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