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Supporting Information For:

Synergistic eradication of antibiotic-resistant bacterial biofilms in vivo with a NIR-sensitive nanoplatform

Kai Dong [a], Enguo Ju [ab], Nan Gao [a], Zhenzhen Wang [ab], Jinsong Ren, * [a] and Xiaogang Qu * [a]

[a] State Key laboratory of Rare Earth Resources Utilization and Laboratory of Chemical Biology, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, China

[b] University of the Chinese Academy of Sciences, Beijing 100039, China

Experimental Section

Reagents and materials

$Gd(NO_3)_3·6H_2O$, $Yb(NO_3)_3·5H_2O$, $TmCl_3·6H_2O$, NH_4F, oleic acid and [2-(Methacryloyloxy)ethyl]trimethylammonium chloride (TMAEMC) were purchased from Aladdin. Tetraethylorthosilicate (TEOS), sodium hydroxide (NaOH) and (3-aminopropyl)trimethoxysilane (APTES) were purchased from Sigma-Aldrich. Hexadecyltrimethylammonium bromide (CTAB) was obtained from Alfa Aesar. Chitosan was purchased from Sangon Biotechnology Co. (Shanghai, China). Drug-resistant *Staphylococcus aureus* strains (ATCC 43300) were obtained from Chuanxiang Biotechnology, Ltd. (Shanghai, China). Ultrapure water was prepared using Milli-Q-Plus system water (18.2 MΩ·cm) and used in all experiments. All reagents were used as received without any further purification.

Preparation of Roussin's Black Salt

Roussin's Black Salt ($[NH_4][Fe_4S_3(NO)_7]$) was prepared according to the work of Seyferth et al. A 500 mL round-bottom flask was charged with 4.5 g of $NaNO_2$ and 20 mL of distilled water. The solid was dissolved, and then 5 mL of a 22% solution of $(NH_4)_2S$ in 15 mL of distilled water was added to the solution. The pale yellow solution was heated at reflux until the solution turned a dark cherry-red. While this solution was being heated, a 250 mL beaker was charged with 10 g of $FeSO_4$, and 80 mL of distilled water. The solid was dissolved to give a pale green-blue solution which was added to the first solution when it had turned deep cherry-red. Instantly, upon addition of the ferrous sulfate solution the reaction mixture turned black. The solution was stirred while it was heated. After 30 s, 12.5 mL of a 22% $NH_3·H_2O$ solution was added in small portions with stirring. The solution was heated at reflux for 10 min and then filtered (hot), quickly through

folded paper towels (filter paper was found to be too fine and would clog almost instantly). The red-brown solid $Fe(OH)_3$, was discarded, and the black-brown solution was allowed to stand overnight. The black crystalline solid was collected and dried under vacuum. The compound was used without further purification. RBS is stable to air in the solid form for several days, however, longer-term storage requires dark and inert atmosphere.

Synthesis of Quaternized Chitosan (qC)

Quaternized chitosan (qC) was obtained by free radical polymerization of chitosan and TMAEMC. Briefly, chitosan (1% w/v) was dissolved in 50 mL of 250 mM acetic acid solution. APS (0.045% w/v) and TMAEMC were added and the mixture was stirred at 60 ^oC under nitrogen stream with a speed of 500 rpm. Mole ratio of $-NH_2$ of chitosan and TMAEMC was 1:1. The reaction was terminated after 2 h and the copolymer solution was dialyzed in demineralized water for 48 h.

Synthesis of UCNPs

25% Yb^{3+}, 0.3% Tm^{3+} co-doped $NaGdF_4$ nanocrystals were prepared as previously reported with some modification. In a typical process, NaOH (1.2 g), water (2 mL), ethanol (8 mL), and oleic acid (20 mL) were mixed under agitation to form a homogeneous solution at room temperature. After then, 1 mL 1 M $Ln(NO_3)_3$, (Ln: 74.7 mol% Gd^{3+}, 25 mol% Yb^{3+}, 0.3 mol% Tm^{3+}) aqueous solution was added under magnetic stirring. Subsequently, 1.0 M aqueous NaF (6 mL) solution was added dropwise to the above solution. After stirring at room temperature for 30 minutes, the mixed reactants were transferred into a 50 mL autoclave, sealed and heated at 200 °C for 12 h. The system was then allowed to cool to room temperature naturally. The resultant nanoparticles were collected by centrifugation at 12000 rpm for 10 min, washed with cyclohexane several times.

Synthesis of UCNPs@mSiO$_2$ nanoparticles

Mesoporous silica coated upconversion nanoparticles (UCNPs@$mSiO_2$) were prepared using a previous method. Briefly, triton (10.8 mL), cyclohexane (45 mL), n-hexanol (10.8 mL), water (2.04 mL), and 200 mg of oleate-capped UCNPs were mixed in a 250 mL flask by sonication and agitation for 40 min. Then 400 μL of TEOS was added dropwise into the mixture. Afterward, 1.2 mL concentrated ammonia aqueous solution (28%) was added into the flask. After stirring for 6 h, the precipitates were separated by centrifugation, washed with ethanol three times, and dried at 60 °C for 12 h. Then, UCNPs@SiO_2 were added into a solution containing tetraethylorthosilicate (TEOS, 100 μL) and octadecyltrimethoxysilane (C18TMS, 40 μL) with a mole ratio of 4.7:1 in a mixture of ethanol (20 mL) and ammonia hydroxide solution (1 mL, 33%) at room temperature and stirred for 6 h. The nanoparticles were collected by centrifugation, washed with ethanol and water 3 times, and then calcined at 500 ^oC for 7 h in air to form mesoporous silica by removal of C18TMS.

Synthesis of RBS@UCNPs@mSiO$_2$@qC

The Roussin's black salt was loaded in the mesoporous silica shells of UCNPs@$mSiO_2$ via an impregnation method. For example, 6 mg of UCNPs@$mSiO_2$ core-shell particles were re-dispersed in 6 mL of ethanol and 2 mg of Roussin's black salt/ethanol solution in the brown vials. After being stirred for 24 h, 1 mL of quaternized ammonium chitosan (qC) (2 mg mL^{-1}) was injected and kept stirring for 3 h. The products were collected by centrifugation and washed with ethanol for 3 times for further use.

Determination of released nitric oxide (Griess assay)

NO release was determined using the two-step process of the Cayman Chemical Nitrate/Nitrite Assay Kit. Diazeniumdiolates readily release NO upon contact with water at physiological pH.

Typically, 20 mg RBS@UCNPs@mSiO$_2$@qC was dissolved in 240 μL of sterile MilliQ water. The solution was incubated at different time points. After incubation, the nanoparticle was separated by centrifuge. The filtrate was mixed with 50 μL of nitrate reductase and cofactor. The solution was incubated for 3 h at room temperature. Following the incubation period wherein the nitrates were converted into the assayable nitrite, 150 μL of each Griess reagent (A & B) were added and the solution incubated for a further 30 min. Nitrite concentrations were then measured using an UV-vis spectrometer. The absorbance at 548 nm was used to calculate the NO content from the assay's calibration curve.

Biofilm growth

Drug-resistant *S. aureus* strain (ATCC 43300) was grown overnight with shaking at 37 °C in Tryptone Soy Broth (TSB) (the concentration of *S. aureus* cells: OD_{600} = 1.0). 10 μL of this *S. aureus* seed solution and 990 μL TSB medium (containing 3% NaCl and 1% glucose) were into the 24-well microtiter plates. Microtiter plates were then incubated without shaking at 37 ^{o}C. The medium was discarded and freshly added every 24 h. After 48 h, each well was washed with PBS buffer under aseptic condition to eliminate medium and unbound bacteria. The generated biofilms could be observed on the bottom of wells.

In vitro Biofilm Dispersion Assays

The obtained biofilms of drug-resistant *S. aureus* were treated with methicillin (MIC>50 μg/mL), RBS@UCNPs@mSiO$_2$, qC, RBS@UCNPs@mSiO$_2$ upon NIR irradiation, and RBS@UCNPs@mSiO$_2$@qC upon NIR irradiation for biofilm, respectively. And the wells containing same concentration of *S. aureus* without any treatments in TSB medium were control groups. After 12h incubation, the medium was removed and the remaining biofilm was washed

once with PBS (1.0 mL). Photographs of the remaining biofilms were obtained using a digital camera.

Biomass Assay

The biofilms were grown in 24-well plates. Wells were stained with 300 µl of 1.0% crystal violet dye, rinsed twice with 2 ml deionized water and thoroughly dried. For quantification, 0.5 ml of 95 % ethanol was added to each well. Plates were incubated for one hour at room temperature with shaking. Crystal violet solution was diluted with 95 % ethanol and the OD at 595 nm was measured using an Ultraspec 2000 (Pharmacia Biotech).

Biofilm Inhibition Assay

The biofilms of *S. aureus* were developed in the 24-well microtiter plates, then treatments (methicilin, qC, RBS@UCNPs@$mSiO_2$ under NIR and RBS@UCNPs@$mSiO_2$@qC under NIR) were implement (Final concentration of *S. aureus*: OD_{600} = 0.01), respectively. Microtiter plates were then incubated at 37 ^oC for 48 h. The generated biofilm was measured by crystal violet staining method as mentioned. And the wells containing same concentration of *S. aureus* without any treatments in TSB medium for 48 h were measured as control groups.

Biofilm dispersal studies using florescence microscopy

For florescence microscopy analysis, sterilized glass slides were put in the bottom of 24-well plates and the growth of biofilm and treatments were incubated as described above. The biofilms were rinsed twice with PBS before being stained with LIVE/DEAD *Bac*Light bacterial viability kit reagents (Molecular Probes) according to the manufacturer's procedure. One microliter of each of the two components was mixed thoroughly in 1 ml of PBS. 0.5 ml of this solution were then trapped by the sample and allowed to incubate at room temperature in the dark for 15 min. After

washed twice with PBS, the samples were observed using an Olympus BX-51 optical system microscopy. Pictures were taken with an Olympus digital camera. Cells that were stained green were considered to be viable and those that stained red were considered to be dead.

Colony-forming units (CFU) assay

The growth of biofilm and treatments were incubated as described above. Then biofilm bacteria were washed once and resuspended in 1 mL PBS by swabbing with a sterile cotton bud and incubating in a sonication bath for 2 mins. Cells were serially diluted in PBS and were placed on LB agar. The agar plates were inverted and incubated at 37 °C for 12 h.

SEM observation

The morphologies of biofilms with or without treatment were observed using a field emission SEM. The biofilms were first grown on the sterilized glass slides, and then were treated with various agents. After treatments, the biofilms were washed with PBS and then fixed in PBS containing 5% formaldehyde for half an hour. The biofilms were further washed with D.I. water, followed by dehydration using a series of ethanol washes and drying at room temperature. The samples were vacuum coated with platinum prior to SEM analysis.

MTT Assays for cytotoxicity

MTT assays were used to probe cellular viability. HeLa cells were seeded at a density of 5000 cells $well^{-1}$ (100 μL total volume per well) in 96-well assay plates. After 24 h incubation, the prepared simples, at the indicated concentrations, were added for further incubation of 48 h. To determine toxicity, 10 μL of MTT solution (BBI) was added to each well of the microtiter plate and the plate was incubated in the CO_2 incubator for an additional 4 h. Then the cells were lysed by the addition of 100 μL of DMSO. Absorbance values of formazan were determined with Bio-

Rad model-680 microplate reader at 490 nm (corrected for background absorbance at 630 nm). Six replicates were done for each treatment group.

Cytotoxic studies using florescence microscopy

For observation by fluorescence microscopy, HeLa cells (1×10^5 cells) were co-cultured with prepared nanoparticles (0.2 mg mL^{-1}) for 6 h in DMEM. After incubation, the cells were then washed three times with PBS and treated with 1 μL calcein AM and 1 μL propidium iodide (PI) for 15 min. After washed twice with PBS, the cells were observed using an Olympus BX-51 optical system microscopy. Pictures were taken with an Olympus digital camera.

In vivo biofilm model of S. aureus biofilm adhered catheter in rats

Wistar rats of 100 g body-weight were purchased from Medical Experimental Animal Center of Jilin University (Changchun, China). Mice handling and procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee. To prepare implants, commercial Vacutainer 21G × 3/4'' × 7'' catheters (Becton-Dickinson) were cut into 10 mm segments. Cleaning and disinfection were achieved thereafter by immersion in ethanol. Sterility was checked by incubation (37 ºC, 24 h) in TSB. Then, biofilm adhered catheter was successfully achieved by incubation (37 ºC, 48 h) in 1 mL TSB containing 1×10^5 CFU of *S. aureus*. Finally, catheters were rinsed with fresh TSB and immediately implanted subcutaneously through a minimal surgical incision in the inner thigh of mice, previously anaesthetized by intraperitoneal administration.

In vivo Biofilm Dispersion Assays

Following *S. aureus* biofilm adhered catheter implantation put into the rats (n=5), the nanocompounds (1 mg/mL, 0.5 mL) were subcutaneously injected in the implanted site. Then, the

implanted site was irradiated by 980 nm NIR (1 W, 5 min). As a control group, the biofilm adhered catheters were firstly implanted into the rats (n=5). Subsequently, the rats were subcutaneously administrated with PBS. After 7 days culture, the rats were euthanatized and implanted catheters were carried out. The eradication of biofilms and the prevention of biofilm-associated infections were evaluated by SEM and histological analysis.

Apparatus and Characterization

SEM images were recorded using a field emission scanning electron microscope (FESEM, S-4500, Hitachi). Transmission electron microscope (TEM) measurements were carried out on a FEI TECNAI G^2 20 high-resolution transmission electron microscope with a tungsten filament at an accelerating voltage of 200 kV. The samples were prepared by placing a drop of prepared solution on the surface of a copper grid and dried at room temperature. UV-Vis spectroscopy was carried out with a JASCO V-550 UV-Vis spectrometer. FT-IR characterization was carried out on a BRUKE Vertex 70 FT-IR spectrometer. The samples were thoroughly ground with exhaustively dried KBr. For the ICP-OES measurements, the samples were firstly treated with the concentrated nitric acid and then were carried out with the Thermo Scientific iCAP6300. Zeta potential was carried out with the Zetasizer Nano ZS 90 (Malvern). The UC spectra was obtained by using a 976 nm laser diode (BWT Beijing LTD.) as the excitation source and recorded on an Edinburgh Instruments FLS920 fluorescence spectrometer.

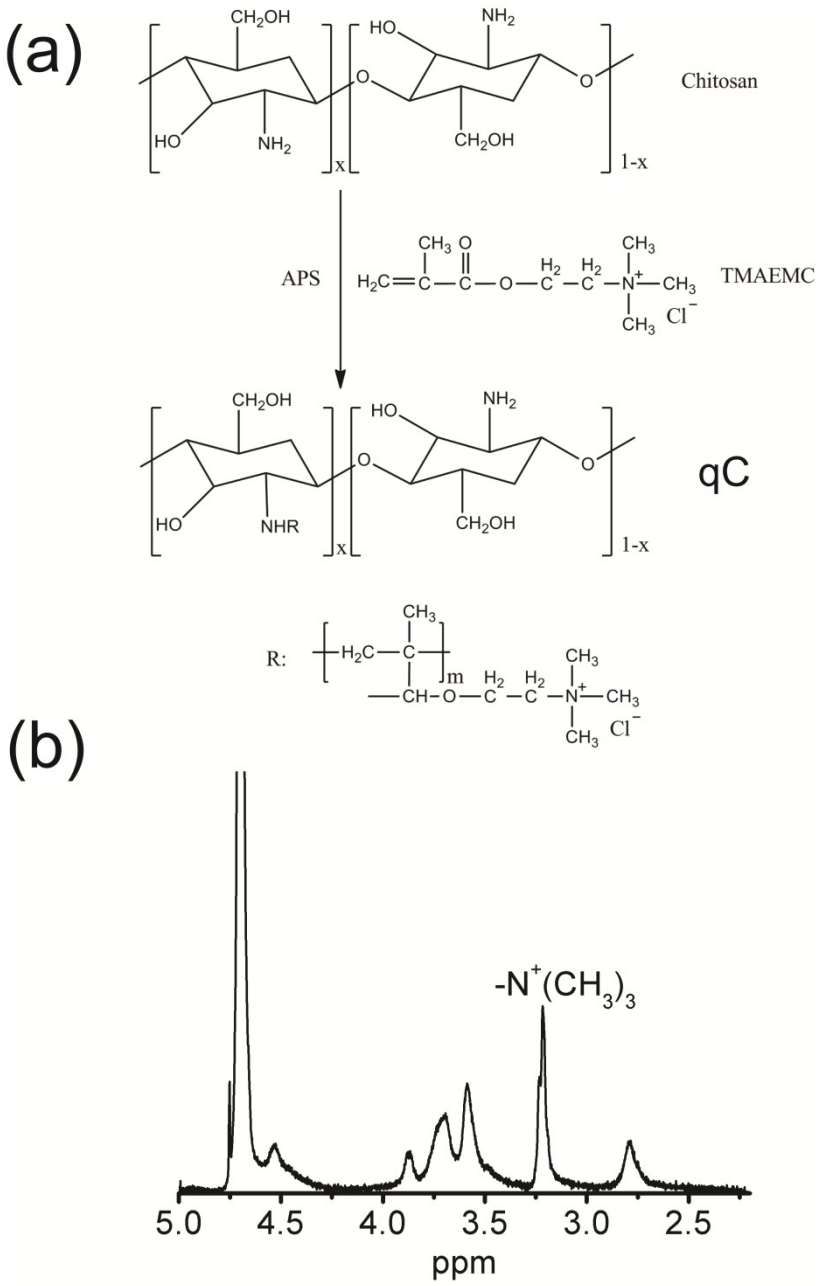

Figure S1. (a) The synthetic scheme of qC and (b) the ^{1}H NMR spectra of qC in D_2O.

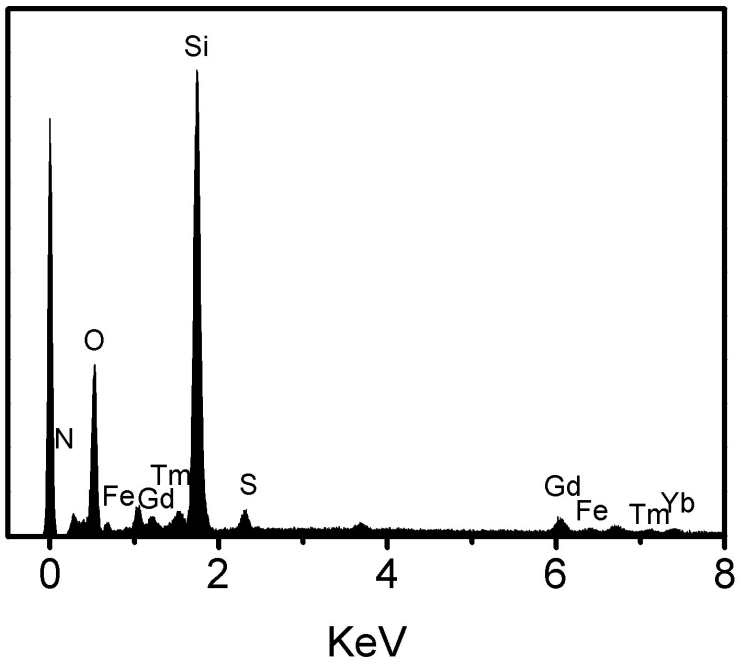

Figure S2. EDS spectrum of RBS@UCNPs@mSiO_2@qC.

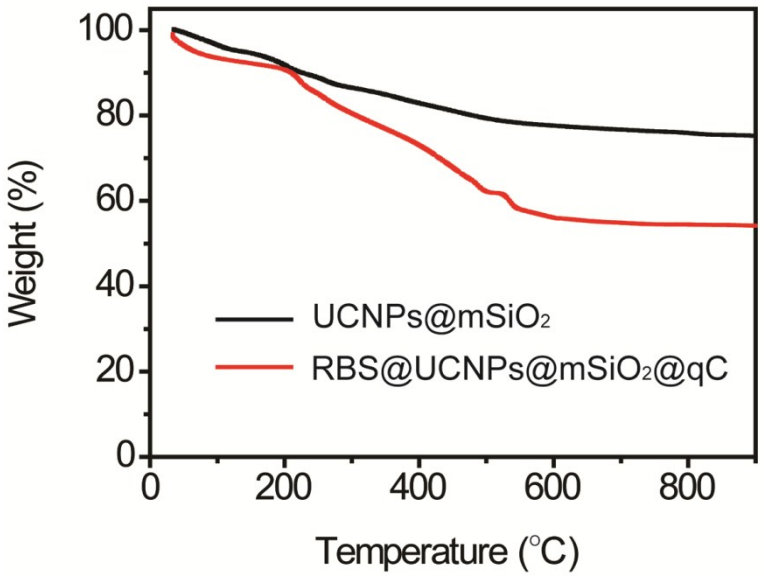

Figure S3. Thermogravimetric analysis (TGA) curves of UCNPs@$mSiO_2$ and RBS@UCNPs@$mSiO_2$@qC.

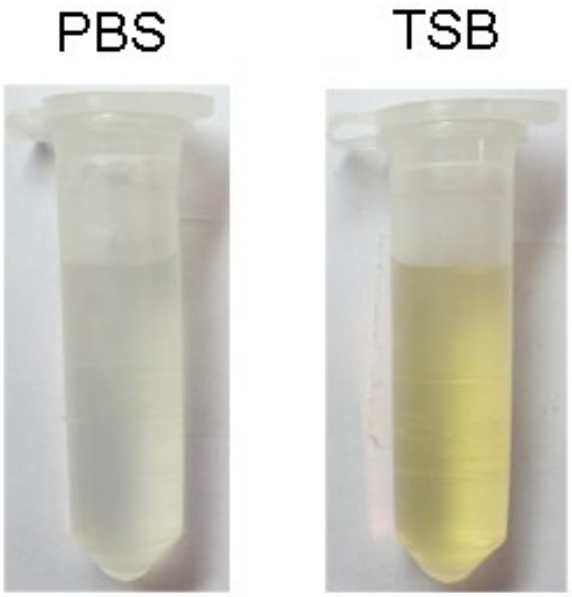

Figure S4. Photos of RBS@UCNPs@mSiO_2@qC nanoparticles dispersing in phosphate buffered saline (PBS) and TSB medium.

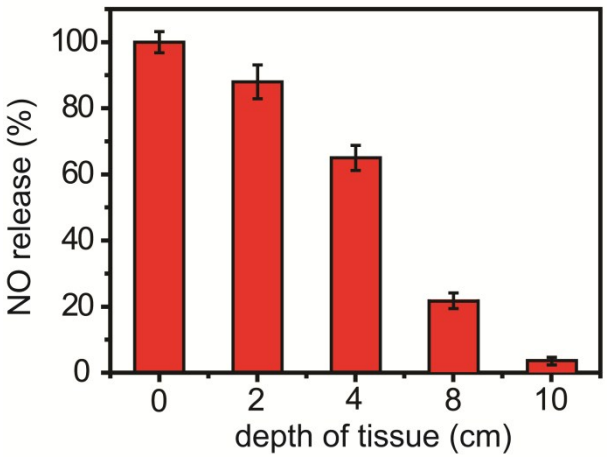

Figure S5. NO release induced by 980 nm NIR laser irradiation through different thicknesses of pork tissue.

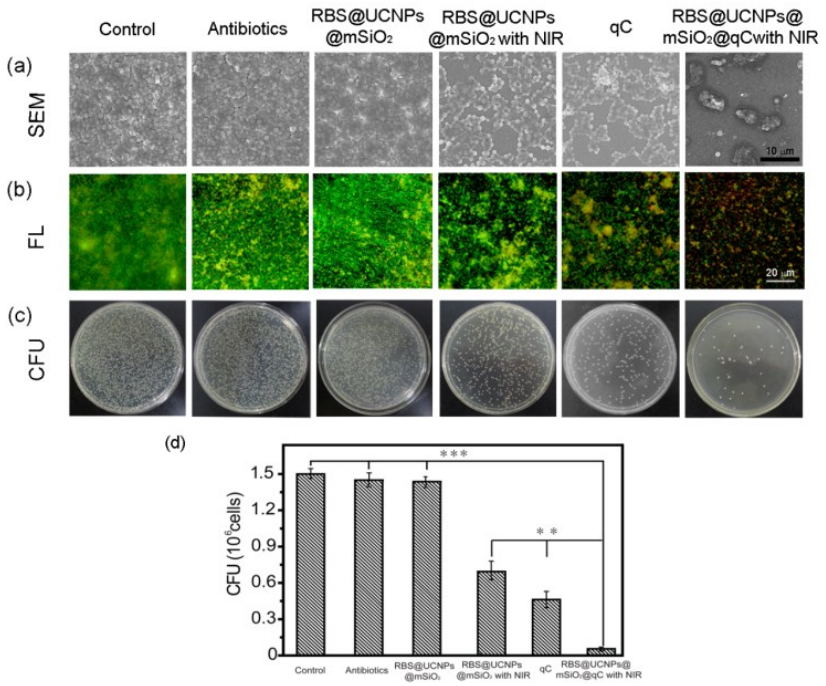

Figure S6. (a) SEM, (b) florescence (FL) microscopy and (c) colony-forming units (CFU) images of synergistic eradicating antibiotic-resistant bacterial biofilms. (d) The quantification of colony-forming units (CFU) in the biofilms after treatment with various agents (**$p < 0.01$ and *** $p < 0.001$).

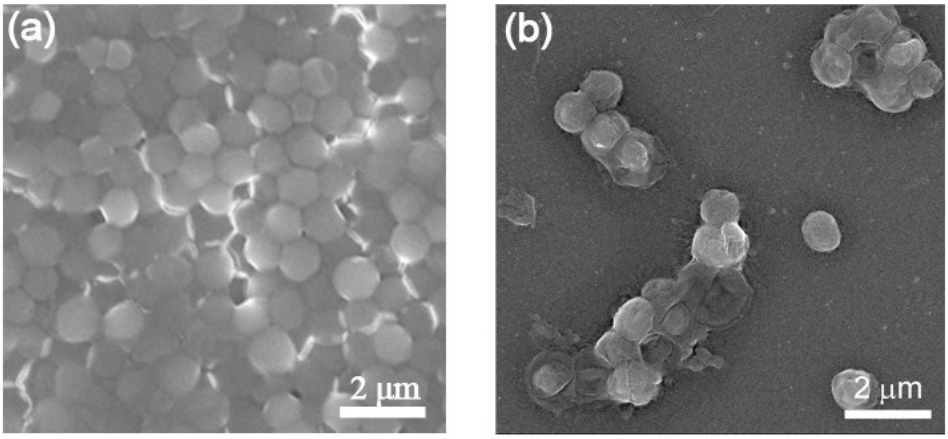

Figure S7. SEM images of (a) bacteria in the biofilms and (b) bacterial deformation and surface roughness in the biofilms after treatment with RBS@UCNPs@mSiO_2@qC upon NIR irradiation.

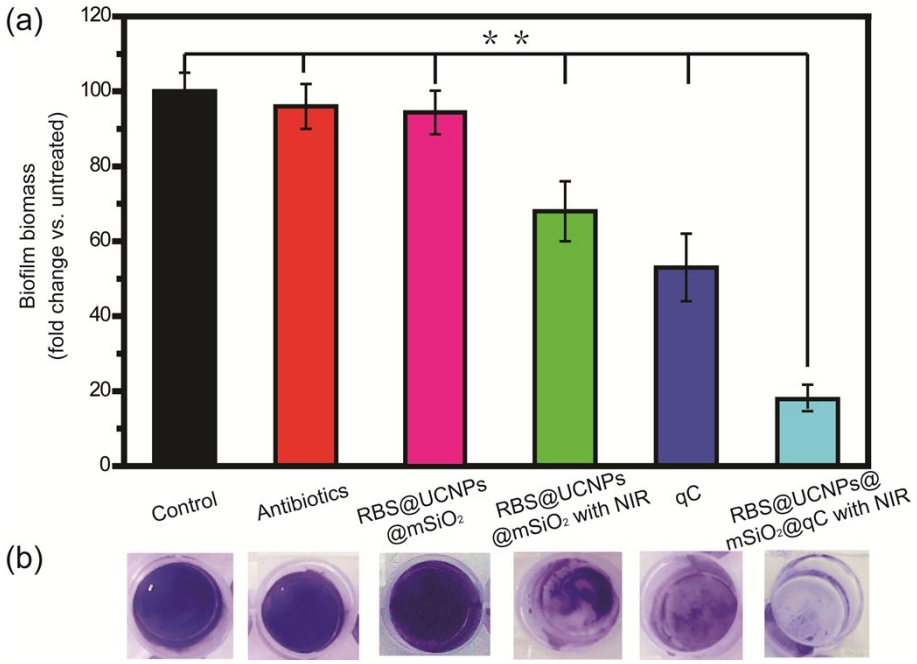

Figure S8. (a) Quantifying prevention of biofilm formation by crystal violet staining treatment with various agents. (b) The photograph of stained biofilms treated with various agents. Dose: antibiotics (10 μg/mL), RBS@UCNPs@$mSiO_2$ (200 μg/mL) with 1 W NIR, qC (40 μg/mL), RBS@UCNPs@$mSiO_2$@qC (200 μg/mL) with 1 W NIR (**$p < 0.01$).

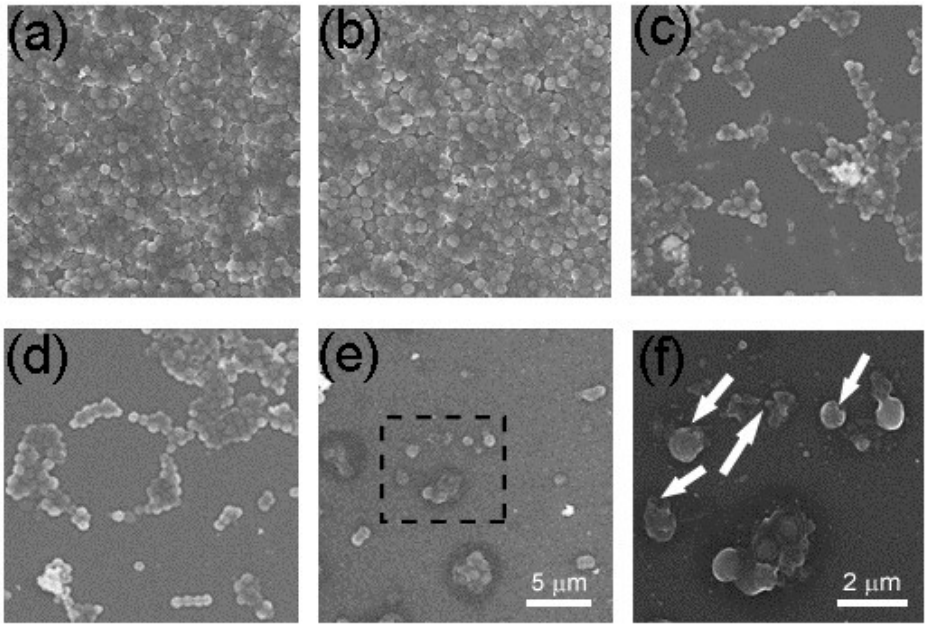

Figure S9. SEM images of prevention of biofilm formation treatment (a) without any agents or with (b) antibiotics, (c) RBS@UCNPs@mSiO$_2$ with NIR, (d) qC and (e) RBS@UCNPs@mSiO$_2$@qC with NIR. (f) Enlarged SEM image of bacterial deformation and membrane lysis after treatment with RBS@UCNPs@mSiO$_2$@qC with NIR (white arrows).

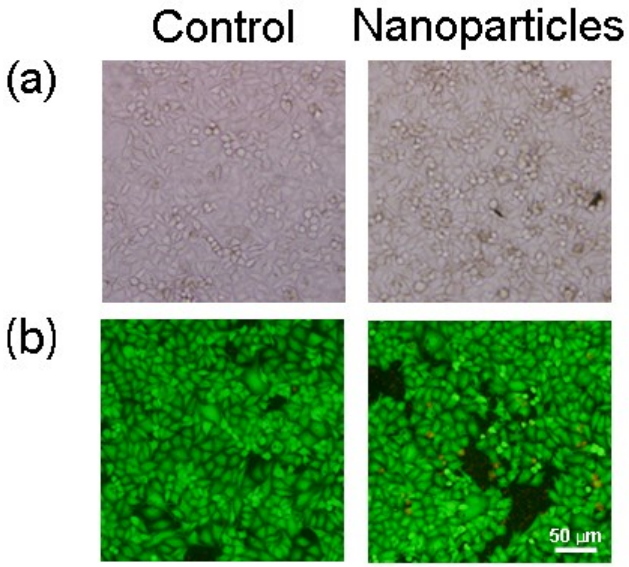

Figure S10. (a) Microscope images of the HeLa cells incubated without and with RBS@UCNPs@mSiO$_2$@qC nanoparticles for 12 h. (b) Fluorescence microscopy images of HeLa cells incubated without and with RBS@UCNPs@mSiO$_2$@qC nanoparticles for 12 h. Viable cells were stained green with calcein AM, and dead cells were stained red with propidium iodide (PI).

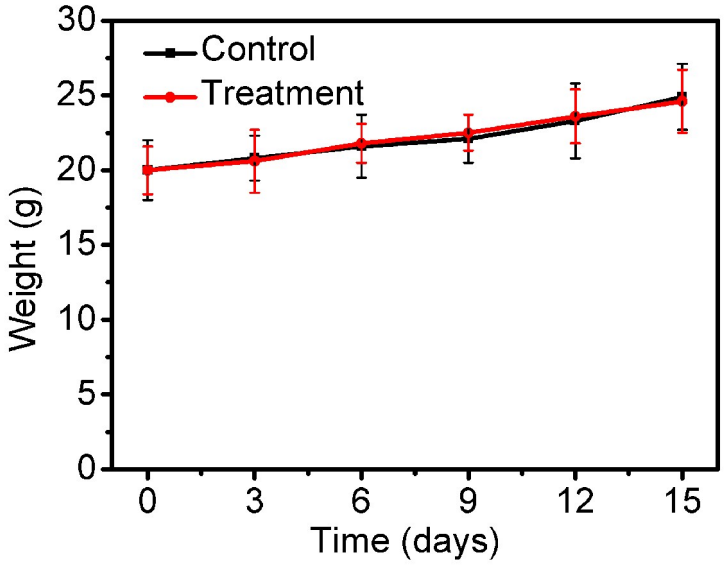

Figure S11. Change in body weight obtained from mice injected with RBS@UCNPs@mSiO$_2$@qC nanoparticles and without injection.

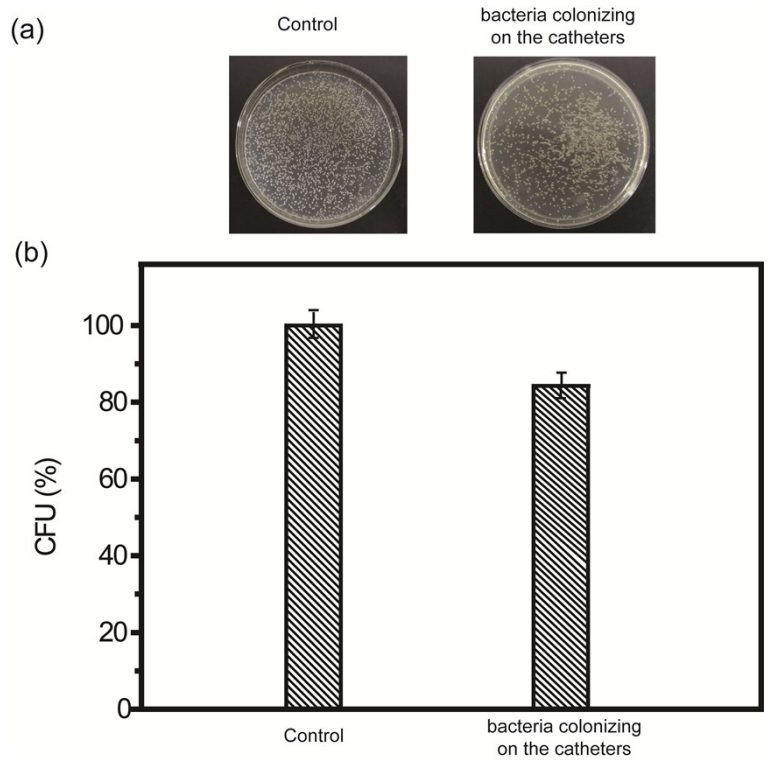

Figure S12. (a) The colony-forming units (CFU) images and (b) the quantification of colony-forming units (CFU) of bacteria colonizing on the catheters compared with the control group (without catheters at the same concentration of liquid strain).