Functionalized Nanoparticles Complexed with Antibiotic Efficiently Kill MRSA and Other Bacteria

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Supporting Information

Experimental Section

Materials. All chemicals were obtained from Fisher or Acros and used as-received unless otherwise specified. 4-Cyanopentanoic acid dithiobenzoate (CPDB) was obtained from Strem Chemical Inc. CPDB immobilized silica nanoparticles were synthesized according to the literature.[1] 3-Aminopropyldimethylethoxysilane was obtained from Gelest and used as-received. NBD based fluorescent dye was prepared according to the literature.[2] *Tert*-butylmethacrylate (99%, Acros) and methacrylic acid (99.5%, Acros) were purified by passing through an activated neutral alumina column. AIBN was recrystallized from methanol before use. The beta-lactam antibiotic penicillin-G potassium salt was obtained from Sigma-Aldrich Inc. and used as-received.

Bacterial Strains. List of organisms used: *Staphylococcus aureus* ATCC 25423; *Bacillus cereus* ATCC 11778; *Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853; *Proteus vulgaris* ATCC 29905; *Salmonella typhimurium* ATCC 13311; *Enterobacter aerogenes* ATCC 13048; *Staphylococcus aureus* ATCC 29213 (community acquired-MRSA); *Staphylococcus aureus* ATCC BAA 1717[3] (hospital associated-MRSA); *Klebsiella pneumoniae* ATCC 13883. The Gram-negative bacterium strain *Escherichia coli* M8820 was obtained from the American Type Culture Collection (ATCC #25922). All the bacterial strains were grown at 37°C in tryptic soy broth (TSB, 30 g/L).

Instrumentation. ^{1}H NMR (Varian Mercury spectrometer 300/400) was conducted using CD_3OD or $CDCl_3$ as the solvent. Molecular weights and PDI were determined using a gel permeation chromatography (GPC) equipped with a 515 HPLC pump, a 2410 refractive index detector, and three Styragel columns. The columns consisted of HR1, HR3 and HR4 in the effective molecular weight ranges of 100-5000, 500-30000, and 5000-500000, respectively. The GPC used THF as eluent at 30 °C and a flow rate of 1.0 mL/min and was calibrated with poly(methyl methacrylate) or polystyrene standards obtained from Polymer Laboratories. Samples were filtered through microfilters with a pore size of 0.2 μm before injection. Infrared spectra were recorded with a PerkinElmer Spectrum 100 spectrometer. UV-vis spectra were measured with a Perkin-Elmer Lambda 4C UV-vis spectrophotometer. TEM images were examined using a Hitachi 8000 transmission electron microscope with an operating voltage of 200 kV. Carbon-coated copper grids were used to prepare samples by dropping sample solutions on the grids followed by drying in a fume hood before use. Tapping mode AFM measurements were operated using a Multimode Nanoscope III system (Digital Instruments, Santa Barbara, CA). The characterization was conducted using commercial Si cantilevers with a spring constant and resonance frequency of 20–80 N m^{-1} and 230–410 kHz respectively. The

sample of PMAA grafted nanoparticles was prepared via spin-coating on silicon wafers with a speed of 3000 rpm. TGA measurement was conducted using a TA Instruments Q5000 with a heating rate of 10°C/min form 30°C to 900°C under nitrogen flow. Dynamic light scattering (DLS) was conducted using a Brookhaven BI-200 goniometer, BI-9000AT correlator (Brookhaven Instruments) with Ar-ion laser (Lexel model 95; λ = 514.5 nm). The measurements were calibrated with a standard of 21 nm polystyrene nanoparticle and conducted five times for each test. The CONTIN approach was used to analyze the experimental data (intensity and size distribution).

Linking Antibiotics to Nanoparticles. To complex Penicillin G (PenG) to nanoparticles, 0.5 ml of dye-labeled monolayer carboxylic acid coated nanoparticles (12.8 mg/ml) and 1.5 mL DI water were mixed. Then, 5 mg PenG was added and incubated at 28 ^{o}C with shaking (200 rpm/min) for 3 hours. The nanoparticle-PenG complex sample was collected by Amicon centrifuge tubes. For poly(methacrylic acid) grafted nanoparticles (22 mg/ml), the same procedure was performed.

Disk-Diffusion Assays. The above listed bacterial strains were grown in tryptic soy broth (TSB, 30 g/L) at 37 ^{o}C overnight with vigorous agitation. Growth of the cultures was assayed by measuring the optical density (600 nm) using absorbance spectrophotometry (Shimadzu, Inc). 0.1 mL of the diluted culture was used to inoculate an agar plate. 6mm disks were put on the plate surface, and then a free PenG or PenG-nanoparticle complex solution were added to the disks at different doses. The plates were allowed to incubate at 35 ^{o}C and the clear zone surrounding a disk represents the areas that bacteria were killed. The bare sNP (Entry 1, Table S1), 100% fluorescent dye-surface functionalized sNP (Entry 5, Table S1), and dye-labeled monolayer carboxylic acid and poly(methacrylic acid) functionalized sNPs were also used to test the toxicity to bacteria using the above-mentioned methods.

A minimum inhibitory concentration (MIC) for all bacteria tested was calculated using reported protocols by determination of the inhibition area (measuring the diameter and the depth of the agar).[4,5]

Determination of Beta-Lactamase Activity. Bacteria were tested in order to verify the presence of the antibiotic-degrading enzyme, beta-lactamase. Beta-lactamase was detected chromogenically in the presence of nitrocefin disks. TSB containing 6 μg PenG (both free PenG and nanoparticle-complexed PenG) was inoculated with *E. coli* and cultured at 37 ^{o}C with shaking at 180 rpm. After 24h, the cell pellets were harvested by centrifuge at 10, 000 rpm for 10 min. 10 μL of each cell pellet was then applied on the nitrocefin disks at room temperature under dark environment. The treatments included free PenG (control group), complexed PenG on monolayer carboxylic acid coated sNPs, and complexed PenG on carboxylated polymer grafted sNPs.

Confocal Scanning Laser Microscopy (CSLM) Imaging

The bacterium *Staphylococcus aureus* was grown in tryptic soy broth (30 g/L) at 37°C in the presence of free PenG, complexed PenG on monolayer carboxylic acid coated sNPs and complexed PenG on carboxylated polymer grafted sNPs, or without any addition. After 18 hours of growth, confocal scanning laser microscopy (CSLM) was used to image cells and determine their viability based on membrane integrity. Cells were stained with Live/Dead BacLight Bacterial Viability Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The Live/Dead BacLight Bacterial Viability Kit consisted of SYTO 9 (green fluorescence, ex 480 nm /em 500 nm) and propidium iodide (red fluorescence, ex 490 nm / em 635 nm). Live cells with intact membranes appeared green and dead cells with disrupted membranes appeared red. CSLM was conducted with a

Leica SP5 CSLM System (Leica Microsystems, Buffalo Grove, IL) equipped with four excitation lasers (405, 488, 594, and 633 nm) and a 63x oil objective.

Table S1. Dye-labeled monolayer carboxylic acid coated nanoparticles.

Entry	Total surface density (groups/nm^2)	Total surface density-equivalent (μmol/g)	Dye in the surface groups (mol%)	Carboxylic Groups/Nanoparticle
1	0	0	0	0
2	0.24	56.91	4.80	287
3	0.40	93.53	3.05	487
4	0.65	154.14	3.40	788
5	0.24	56.91	100	0

Table S2. MICs of penicillin (PenG)-complexed nanoparticles and free-penicillin (PenG-Ctrl) for various Gram-positive and Gram-negative bacterial pathogens.

Pathogen Strain	MIC (μg/ml) PenG-Ctrl	PenG-Monolayer	PenG-Polymer
Gram-Positive:			
Staphylococcus aureus	20.3	5.7	4.9
Bacillus cereus	6.9	6.0	3.6
Gram-Negative:			
Escherichia coli	13.5	10.9	5.0
Klebsiella pneumoniae	20.8	18.2	18.2
Proteus vulgaris	41.5	18.2	17.1
Enterobacter aerogenes	17.2	11.5	7.6
Salmonella typhimurium	10.7	5.7	4.3
CA-MRSA	45.9	36.4	20.8
HA-MRSA	4.8	3.5	2.9

Note: The MIC values for a variety of bacteria were calculated using reported protocols by determination of the inhibition area (measuring the diameter and the depth of the agar).[4,5]

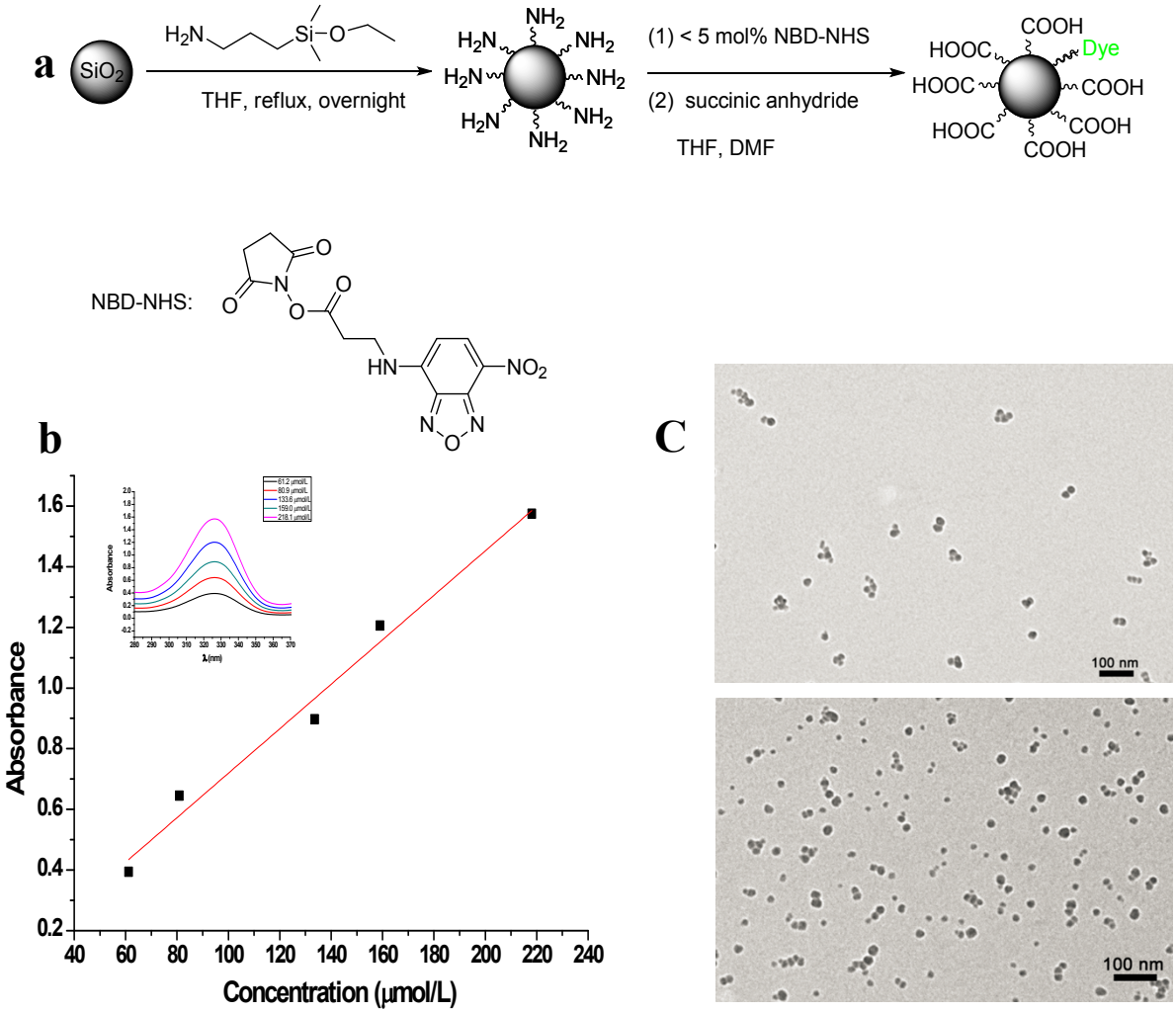

Figure S1. Dye-labeled monolayer carboxylic acid coated silica nanoparticles (sNPs). **a,** Scheme for synthesis of dye and carboxylic acid functionalized nanoparticles. **b**, UV-vis standard absorption curve of NBD-COOH at 326 nm. **c**. TEM images of (A) amine functionalized silica nanoparticles and (B) dye-labeled carboxylic acid functionalized silica nanoparticles.

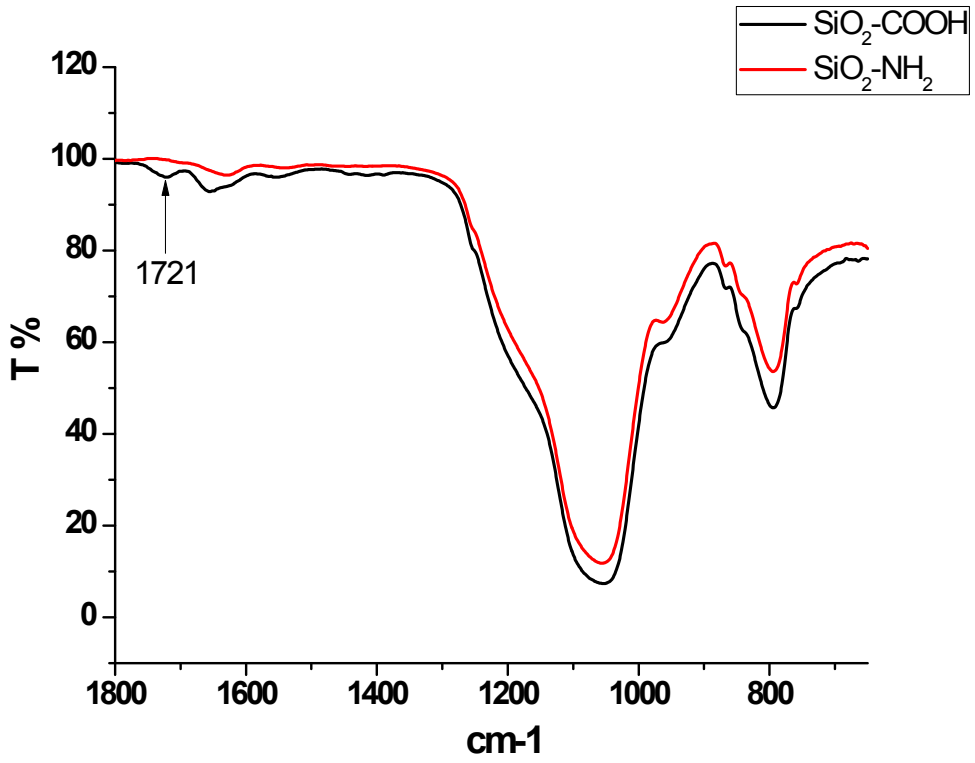

Figure S2. IR spectra of amine functionalized silica nanoparticles (SiO_2-NH_2) and carboxylic acid functionalized silica nanoparticles (SiO_2-COOH).

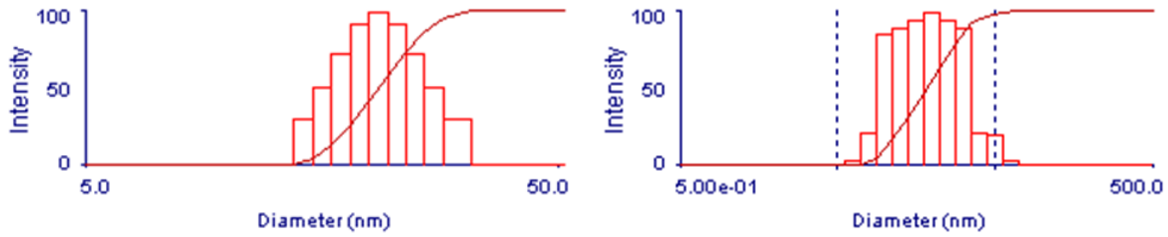

Figure S3. Size distribution of nanoparticles: SiO_2-COOH (left, average diameter of 22.7 nm based on replicate (5x) parallel tests); (b) Bare SiO_2.(right, average diameter of 18.9 nm based on replicate (5x) parallel tests).

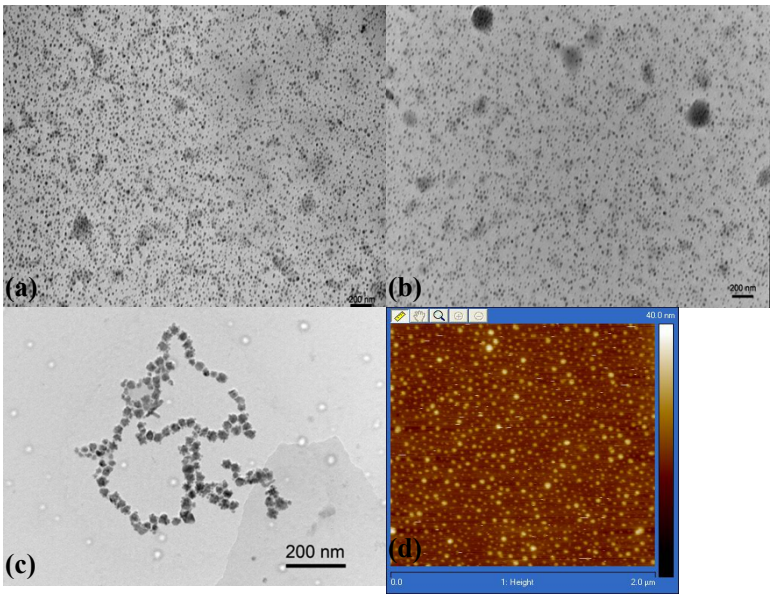

Figure S4. TEM and AFM image of polymer grafted sNPs: (a) poly(tert-butyl methacrylate) grafted sNPs with RAFT group chain end capped; (b) poly(tert-butyl methacrylate) grafted sNPs without RAFT group chain end capping; (c) PMAA grafted sNPs. Scale bar for all the TEM images: 200 nm. (d) AFM image of PMAA grafted sNPs.

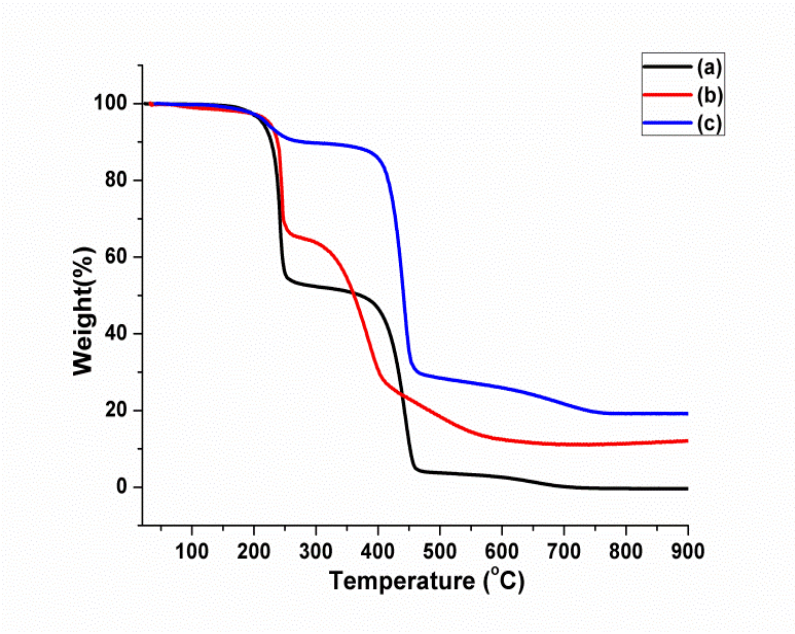

Figure S5. TGA in nitrogen of polymer grafted sNPs: (a) poly(*tert*-butyl methacrylate), Mn=7000 g/mol; (b) poly(*tert*-butyl methacrylate) grafted sNPs without RAFT group chain end capped); (c) PMAA grafted sNPs.[6]

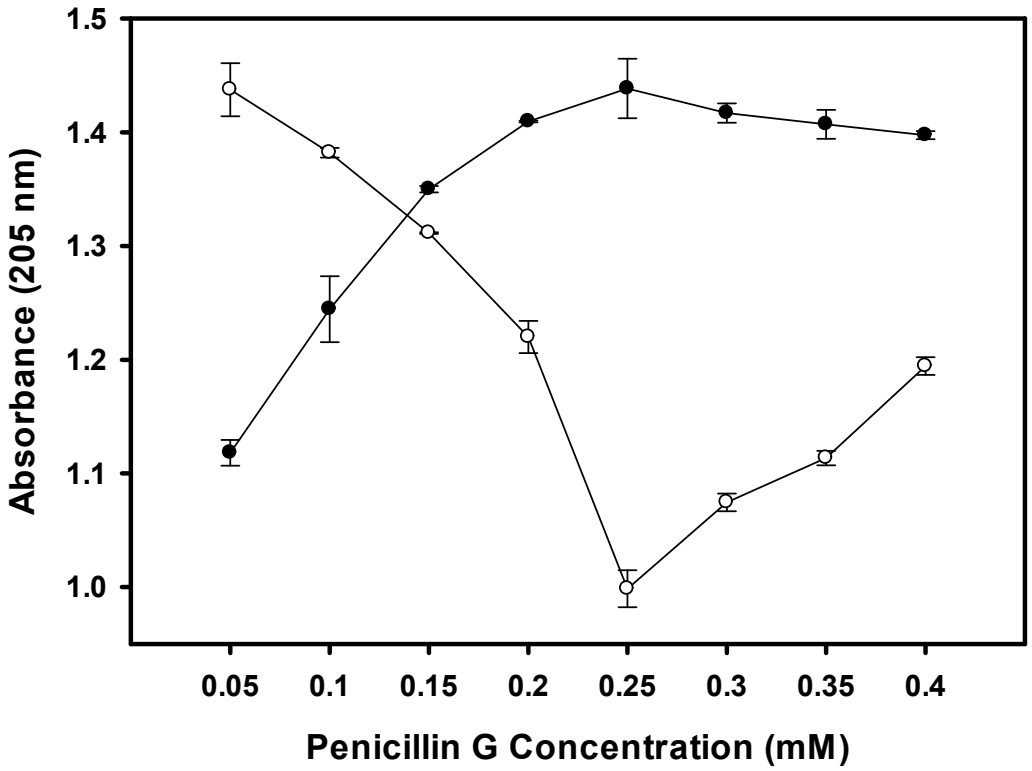

Figure S6. Concentration of penicillin G after separation of PenG-nano complex from free PenG via Amicon Ultra Centrifuge Filters. Starting concentration of penicillin varied from 0.05 mM to 0.4 mM while concentration of poly(methacrylic acid) grafted nanoparticles was held constant at 0.025 mM. Pellet (●) represents nanoparticle complex collected on Amicon filter; supernatant (○) represents free penicillin G eluted through filter.

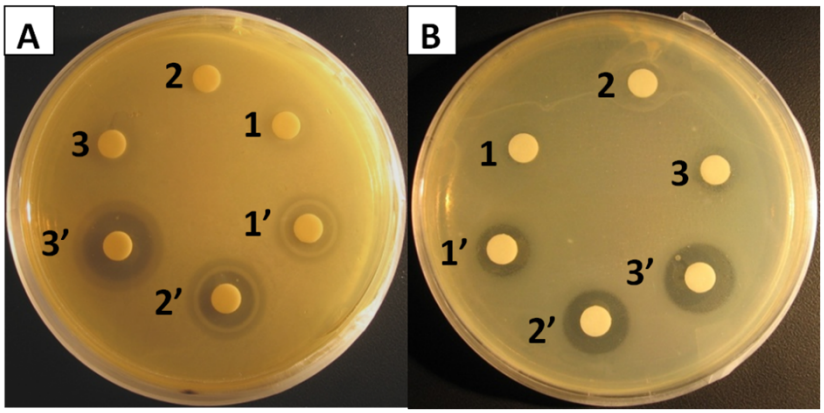

Figure S7. Results of disk-diffusion assays using (A) *S. typhimurium* and (B) *E. coli.* Disks 1, 2, and 3 represent 2.5, 5.0, and 7.5 µg of PenG added in soluble form, respectively. Disks 1', 2', and 3' have 2.5, 5.0, and 7.5 µg of PenG complexed to monolayer surface-carboxylated silica nanoparticles (sNPs), respectively. Activities against both bacterial strains are enhanced significantly when antibiotics are complexed to sNPs.

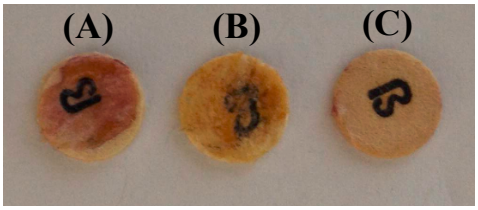

Figure S8. Results of nitrocefin disk assays: (A) treated with the pellet from the incubation of free PenG and *E. coli.*; (B) treated with the pellet from the incubation of monolayer carboxylic acids based nanoparticle-PenG complex and *E. coli.*; and (C) treated with the pellet from the incubation of PMAA based nanoparticle-PenG complex and *E. coli.*

References

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