

Colorimetric Screening of Bacterial Enzyme Activity and Inhibition Based on the Aggregation of Gold Nanoparticles

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Experimental Procedure:

1. Material:

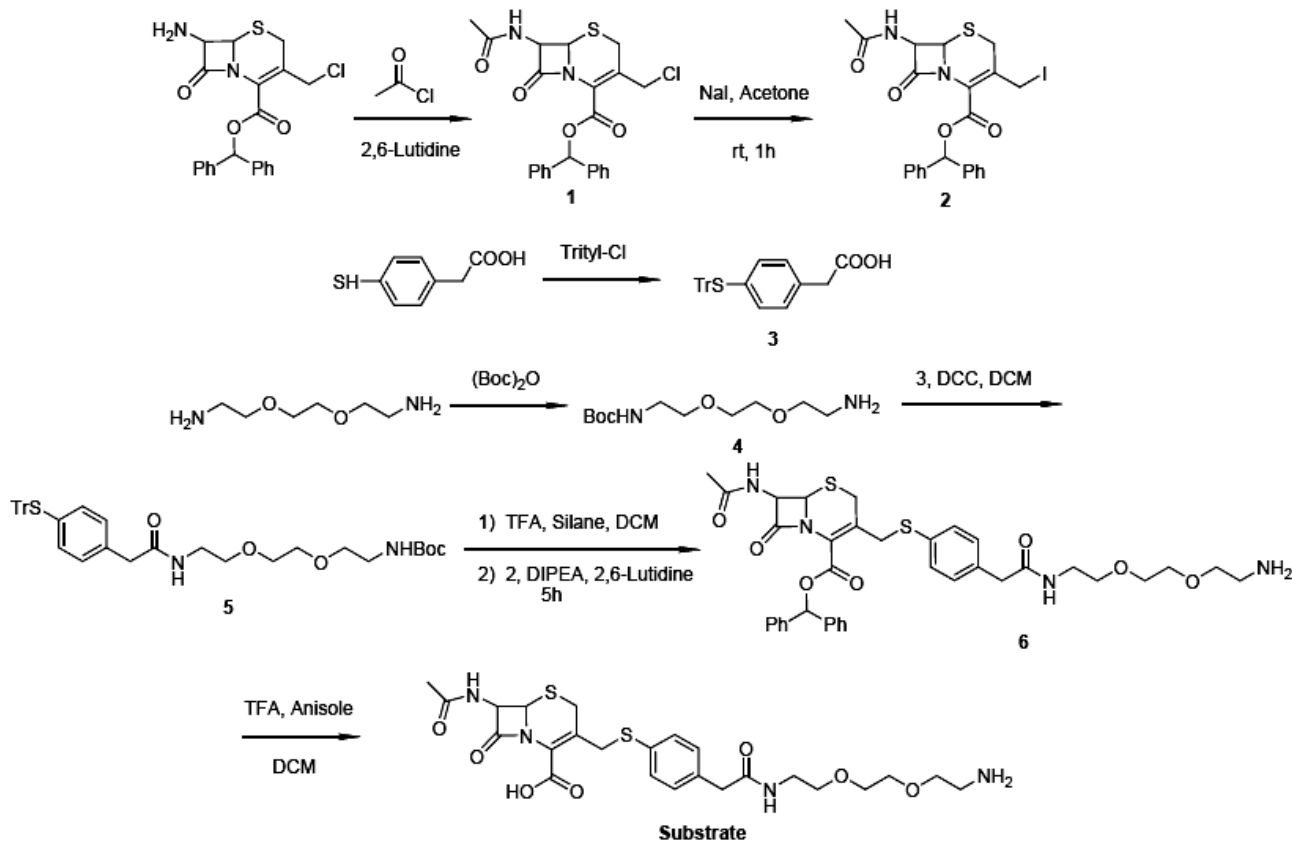
Chemicals: 7-Amino-3-chloromethyl 3-cephem-4-carboxylic acid diphenylmethyl ester hydrochloride (ACLH) was provided from Otsuka chemical Co. Ltd. Nitrocefin was purchased from Merck. β -lactam resistant *K. pneumoniae* bacterial strain (ATCC 700603), TEM-1 *E. coli* strain (ATCC 35218) and *Bacillus cereus* strain (ATCC 13061) were purchased from ATCC. The purified transformed TEM-1 β -lactamase was obtained from Biologics Process Development, CA, USA. All the other starting materials were obtained from Sigma or Aldrich. Commercially available reagents were used without further purification, unless noted otherwise. The solvents were dried according to regular protocols. All other chemicals were analytical grade or better.

The synthesized compounds were characterized by using ¹H NMR (Bruker Advance 400MHz) using CDCl₃ as the solvent. ESI-MS spectrometric analyses were performed at the Thermo Finnigan LCQ Deca XP Max and transmission electron micrograph on a JEOL 2000 EX TEM. Absorbance spectra were measured on Beckman Coulter DU 800 UV-Vis spectrophotometer. HPLC experiments were conducted on Shimadzu LC-20A.

Purification and preparation: Analytical reverse-phase high performance liquid chromatography (HPLC) was performed on Alltima C-18 column (250×3.0 mm) at a flow rate of 1.0 mL/min and semi-preparative HPLC was performed on the similar C-18 column (250×10 mm) at a flow rate of 3 mL/min. An eluting system consisting of A (water with 0.1% TFA) and B (acetonitrile with 0.1% TFA) was used under a linear gradient to elute the products, which was monitored by UV-Visible absorbance at 280 nm. The linear gradient started from 80% solution A and 20 % solution B, changed to 20 % solution A and 80 % solution B in 30 minute and to 0 % solution A and 100% solution B in the following 5 minutes, and then back to 80 % solution A and 20 % solution B in the next 5 minutes.

2. Synthesis and characterization of enzyme substrate.

Enzyme **substrate** was prepared according to **scheme S1**.



Scheme S1

Preparation of 1: 7-Amino-3-chloromethyl cephalosporanic acid benzylhydriyl ester hydrochloride (ACLH) (451 mg, 1 mmol) was suspended in dichloromethane. Then acetyl chloride (78.5 mg, 1 mmol) was added drop wise into the suspension. Finally 2, 6-lutidine (214 mg, 2 mmol) was added into the reaction mixture and the solution was stirred for 2 hrs under nitrogen. After the removal of the solvent on the rotary evaporator, the residue was purified by flash chromatography on silica gel (eluent: ethyl acetate / hexane =1/1) to afford 223.5 mg (yield: 85.4%) of title compound. $^1\text{H NMR}$ (400 MHz, CDCl_3) 7.47-7.45 (m, 2H), 7.41-7.31 (m, 7H), 7.28-7.26 (m, 1H), 6.98 (s, 1H), 5.90 (dd, $J = 8.9$ and 4.92 Hz, 1H), 5.01 (d, $J=4.95$ Hz, 1H), 4.40 (s, 2H), 3.65 (d, $J = 18.3$ Hz, 1H), 3.51 (d, $J = 18.3$ Hz, 1H), 2.02 (s, 3H). ESI-MS observed $[\text{M}+\text{H}]^+$: 457.7, calculated: 456.9.

Preparation of 2: A mixture of **1** (155 mg, 0.34 mmol) and sodium iodide (253 mg, 1.7 mmol) in 5mL of acetone was stirred for 1 hr at room temperature. The reaction mixture was concentrated on the rotary evaporator and diluted with 5 mL water. The suspension was extracted with 25 mL of ethyl acetate, and the organic phase was washed with 10% sodium thiosulfate (5 mL \times 2), brine (5 mL \times 3) and

dried over anhydrous magnesium sulfate. The slightly orange powder **2** (152 mg, 0.45 mmol) was used without further purification.

Preparation of 3: 4-mercaptophenylacetic acid (369.6 mg, 2.2 mmol) was added to the solution of chlorotriphenylmethane (557.6 mg, 2 mmol) in 2.0 mL dichloromethane. The solution was stirred for 2 hrs under nitrogen. The reaction was quenched by 1N NaOH (3 mL). The suspension was extracted with 10 mL of ethyl acetate, and the organic phase was washed with brine (5 mL×3) and dried over anhydrous magnesium sulfate. The solvent was removed and residue was purified by flash chromatography on silica gel (eluent: ethyl acetate/hexane =1/3) to afford 713.4 mg of desired product (87%). ¹H NMR (400 MHz, CDCl₃) δ 7.43-7.40 (m, 6H), 7.26-7.17 (m, 9H), 6.93 (m, 4H), 3.52 (s, 2H) ESI-MS: observed [M+Na]⁺: 433.6, calculated: 410.5.

Preparation of 4: 3,6-Dioxaoctyl-1,8-diamine (244 mg, 1.65 mmol) was stirred in dry dichloromethane (1 mL), then di-*tert*-butyl carbonate (120 mg, 0.55 mmol) in dry dichloromethane (1 mL) was added slowly over 2 hours. The reaction was stirred overnight at room temperature, the solvent evaporated, and the residue purified by column chromatography (silica gel, ethanol:ethyl acetate:triethylamine eluent = 5/4/1). The product was obtained as a pale yellow oil (96 mg, 71%). ¹H-NMR (400 MHz, CDCl₃) δ 3.67 (s, 4H), 3.55-3.62 (m, 4H), 3.34-3.40 (m, 2H), 2.94 (t, *J* = 5.2 Hz, 2H), 1.49 (s, 9H). ESI-MS observed [M+Na]⁺: 271.2, calculated: 248.3.

Preparation of 5: To a cooled (ice bath) and stirred solution of compound **4** (74.4 mg, 0.30 mmol) in 1.0 mL anhydrous dichloromethane was added compound **3** (123.2 mg, 0.30 mmol). Then *N,N'*-dicyclohexylcarbodiimide (123.6, 0.30 mmol) in dry dichloromethane (1 mL) was added slowly over 2 hours. The solution was stirred overnight. The reaction mixture was concentrated on the rotary evaporator. Purification of the crude product by flash chromatography on silica gel (eluent: methanol / dichloromethane = 5/95) afforded the desired product 65.8 mg (60%). ¹H NMR (400 MHz, CDCl₃) δ 7.41-7.38 (m, 6H), 7.27-7.15 (m, 9H), 6.91 (m, 4H), 3.52-3.49 (m, 8H), 3.42-3.27 (m, 4H), 3.27 (m, 2H), 1.43 (m, 9H) ESI-MS: observed [M+Na]⁺: 663.7; calculated: 640.8.

Preparation of 6: To a cooled solution of **5** (43.8 mg, 0.12 mmol) in 0.5 mL of anhydrous dichloromethane was added trifluoroacetic acid (1 mL) and triisopropylsilane (80 μL) with cooling (ice bath). The mixture was stirred for 1 hr at the same temperature, then the solvent was removed under reduced pressure. The residue was washed with cold hexane (1 mL×3) to afford 25.6 mg of the light yellow crude product. The crude product was used for next step reaction without further purification. The product was added drop-wise to a solution of compound **2** (82.3 mg, 0.051, 0.15 mmol) in 0.5 mL

anhydrous *N,N*-dimethylformamide (DMF), followed by addition of *N,N*-diisopropylethylamine (DIPEA, 26.4 μ L, 0.15 mmol) and 2, 6-lutidine (84 μ L, 0.72 mmol). The mixture was stirred at room temperature for 5 hrs. Then, the reaction mixture was diluted with water (5 mL) and extracted by ethyl acetate (10 mL). The organic phase was washed by brine (5 mL) and dried over anhydrous magnesium sulfate. The solvent was removed and the crude product was further purified by RP-HPLC to collect 8.9 mg of compound **6**. ^1H NMR (400 MHz, CDCl_3) δ 7.41-7.28 (m, 10H), 7.13 (d, $J = 8.0$ Hz, 2H), 7.06 (d, $J = 8.0$ Hz, 2H), 6.80 (s, 1H), 5.74 (dd, $J = 8.9$ and 4.92 Hz, 1H), 4.93 (m, 3H), 4.07 (d, $J = 13.2$ Hz, 1H), 3.75 (d, $J = 13.2$ Hz, 1H), 3.63-3.52 (m, 12H), 3.46 (m, 2H), 3.05 (m, 2H), 2.06 (s, 3H). ESI- MS observed $[\text{M}+\text{Na}]^+$: 741.6, calculated: 718.8.

Preparation of substrate: A solution of **6** (3.5 mg, 0.0049 mmol) was dissolved in 150 μ L of anhydrous dichloromethane. Then trifluoroacetic acid (200 μ L) and anisole (9.0 μ L) were added. The mixture was stirred for 1 hr at the cooled temperature (ice bath). The solvent was removed under reduced pressure. The precipitate was collected and washed with hexane (1 mL \times 3) and then purified by RP-HPLC to afford 1.8 mg (65%) of the title product. ESI-MS observed $[\text{M}+\text{H}]^+$: 553.4, calculated: 552.6.

3. Citrate-coated gold nanoparticles.

Gold nanoparticles (15 nm) were prepared by citrate reduction of HAuCl_4 .¹ HAuCl_4 (100ml, 0.25mM, 2.5×10^{-5} mol) was dissolve in 95 ml of deionized water. The aqueous solution was refluxed for 10 min and followed by addition of 5 ml of 0.5% sodium citrate solution. The mixture was refluxed for another 30min until the color of the solution would change gradually from faint yellowish to wine-red. The pH was adjusted to 7.4 by using 0.1 M of NaOH. After filtration through 0.45 μ M Millipore syringe to remove the precipitate, the filtrate was stored at room temperature.

4. Quantitative analysis of β -lactamase induced Au-NP aggregation

Enzyme hydrolysis of substrate by β -lactamase (Bla)

According to the method as reported previously,² all the colorimetric assays mainly consisted of a two-step interaction: one step was the enzymatic hydrolysis to release the thiol group from β -lactam ring. The second one was the binding of released fragments with free thiol terminal and positively charged amino groups to the surface of Au-NPs to induce the aggregation. In a typical experiment, Substrates solutions were prepared in 10 mM of PBS or deionized water. Bla was dissolved in 10 mM

PBS buffer (pH 7.4) and diluted with same PBS buffer to make different Bla concentration. Bla solution (5 μ l) was mixed with substrates in 95 μ l PBS buffer. The final concentrations of Bla and substrate were maintained at 2.0 nM and 8.0 μ M, respectively. The enzymatic reaction was performed by incubating Bla solution with substrate for 20 min at room temperature. All the tests were performed in triplates.

Aggregation test with gold nanoparticles

After 20 min enzyme treatment, the resulting substrate solution was mixed with Au-NPs suspension (15 nm, 400 μ l). As a control, Au-NPs suspension (400 μ l) was mixed with 100 μ l of 10 mM or the diluted PBS buffer solution, and then color change and UV-Vis absorbance change at 650 nm was analyzed every 2 min for 30 min at room temperature by Beckman Coulter DU 800 Uv-Vis spectrophotometer.

In addition, the influence of PBS concentration and pH value for the aggregation of Au-NP was also conducted by mixing Au-NP suspension (400 μ l) with 100 μ l PBS buffer solutions with different concentration or by mixing Au-NP with different concentration of HCl or NaOH, and then color change and UV-Vis absorption spectrum of the Au-NP suspension were analyzed by Beckman Coulter DU 800 Uv-Vis spectrophotometer.

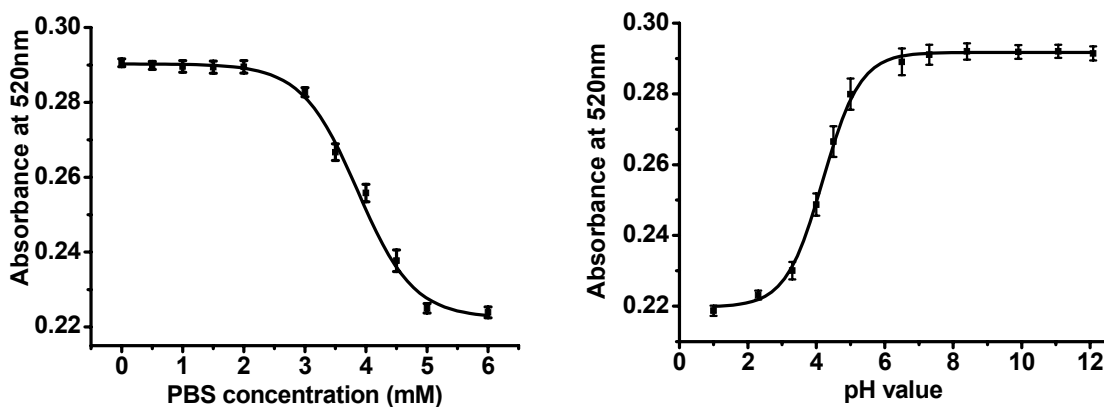


Figure S1. Absorbance change of gold nanoparticles after addition of different concentration PBS buffer solution (left) and absorbance change with different pH value (right).

As shown in Figure S1, Au-NPs showed no aggregation in the PBS buffer with concentration below 3 mM. In addition, Figure S1 also demonstrated that Au-NPs were stable with pH 5.5 or above. In our AuNPs based colorimetric assays, upon the enzymatic hydrolysis and following by mixing the enzyme-pretreated substrate with AuNPs suspension, the final PBS concentration was around 2 mM and pH value was 7.4, which were lower than the threshold of Au-NPs aggregation. Therefore, under

the experimental conditions, both PBS buffer and pH value would not affect Au-NPs aggregation in the process of enzyme interactions.

5. Enzyme detection.

Substrates solutions were prepared in PBS or deionized water and a range of concentrations of Bla were prepared in PBS buffer (pH 7.4). Then Bla solution was mixed with substrates for the enzyme interactions. The substrate concentration was maintained at 8.0 μM . The enzymatic reaction was performed by incubating Bla solution with substrate for 20 min at room temperature. Finally, the mixture was transferred into Au-NPs suspension to induce the aggregation of Au-NPs. The color change and absorbance at 650 nm was measured by Beckman Coulter DU 800 UV-Vis spectrophotometer.

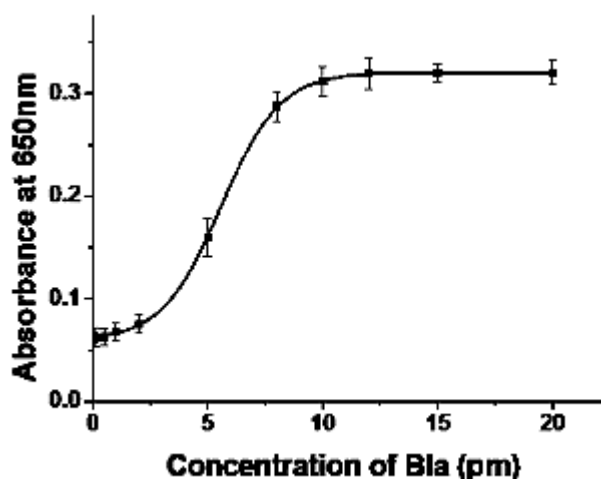


Figure S2. Change in A_{650} of gold nanoparticles with substrate and a range of concentration of transformed TEM-1Bla.

6. Inhibition assay for Bla activity by using gold nanoparticles

For the inhibition assay of Bla activity, the procedure is similar with that in the enzyme reaction for aggregation of Au-NPs. The final concentrations of substrate and Bla solutions were maintained at 8.0 μM and 2.0 nM, respectively. Various β -lactamase inhibitors (2.0 μM , pH 7.4) were mixed with Bla solution first. Then the mixture was incubated at room temperature for 20 minutes to inhibit Bla activity. The inhibitor pre-treated Bla solution was mixed with substrate for 20 minutes. Finally, the substrate solution with inhibitor pre-treated Bla was added into Au-NPs suspension and the mixture was immediately transferred into 96-well plate (Corning Inc., Corning, NY). The color change and

UV-Vis absorption spectra of Au-NPs suspension were collected by Beckman Coulter DU 800 UV-Vis spectrophotometer.

7. Dynamic Light Scattering (DLS) for size distribution in solution

The size population distributions of gold nanoparticles in substrate (8.0 μM) treated Au-NPs suspensions, Bla (2.0 nM) pre-treated substrate (8.0 μM) Au-NPs suspensions with and without inhibitor were determined on a Brookhaven Instruments spectrophotometer. All the measurements were performed at an angle of 90 under room temperature. The CONTIN algorithm was used for analyze the DLS data. DLS analysis presented that the average hydrodynamic diameter of Au-NPs increased significantly from $17.4 \pm 2\text{nm}$ to $52.9 \pm 4\text{nm}$ after the addition of Bla-treated substrate. The size increase demonstrated the enzymatic reaction lead to the aggregation of Au-NPs. While in the presence of sufficient inhibitor, the corresponding DLS measurement demonstrated the average hydrodynamic diameter of Au-NPs of $17.8 \pm 3\text{nm}$, which was almost the same with the value in the Au-NPs suspension without enzyme treatment. These results demonstrated that the interaction between enzyme and substrate was crucial for the aggregation of Au-NPs.

8. Colorimetric assay by using β -lactamase, inhibitors and nitrocefin

Various β -lactamase inhibitors (3.0 μM) were mixed with Bla (2.0 nM) solution first. Then the mixture was incubated at room temperature for 20 minutes to inhibit Bla activity. After that, the inhibitor pre-treated Bla was added into nitrocefin solution. The final concentration of nitrocefin was maintained at 20 μM . Then the mixture was immediately transferred into 96-well plate. After 10 min interaction, the solution was applied for colorimetric image. Using a separate set of samples, UV-Vis absorption spectra were collected by Beckman Coulter DU 800 UV-Vis spectrophotometer.

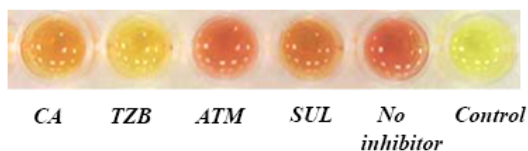


Figure S3. Color of the nitrocefin solution with or without addition of TEM-1 Bla and inhibitors.

9. IC₅₀ detection by using gold nanoparticles and nitrocefin

IC₅₀ values (concentration of inhibitor that reduces enzyme activity to 50% of the activity of the native enzyme) were detected by addition of various β -lactamase inhibitors (TZB, CA, SUL and ATM) with different concentration into Bla solution (2.0 nM). The mixture was incubated for 20 min at room temperature to inhibit Bla activity. The pre-treated Bla solution was mixed with substrate for 20 minutes and then added into Au-NPs suspension to induce the aggregation of Au-NPs. Absorbance change at 650 nm was analyzed by Beckman Coulter DU-800 UV-Vis spectrophotometer. As control, IC₅₀ values of the inhibitors were also determined by standard indicator: nitrocefin.

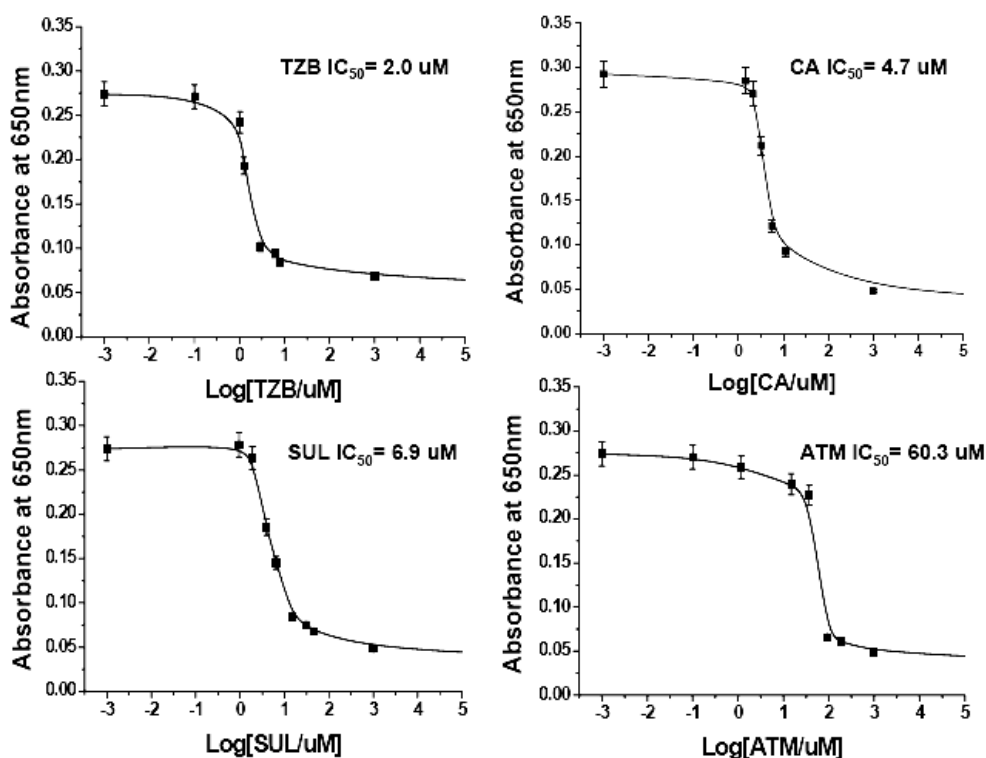


Figure S4. IC₅₀ values of TZB, CA, SUL and ATM for transformed TEM-1 β -lactamase by using substrate (8 μ M) and gold nanoparticles.

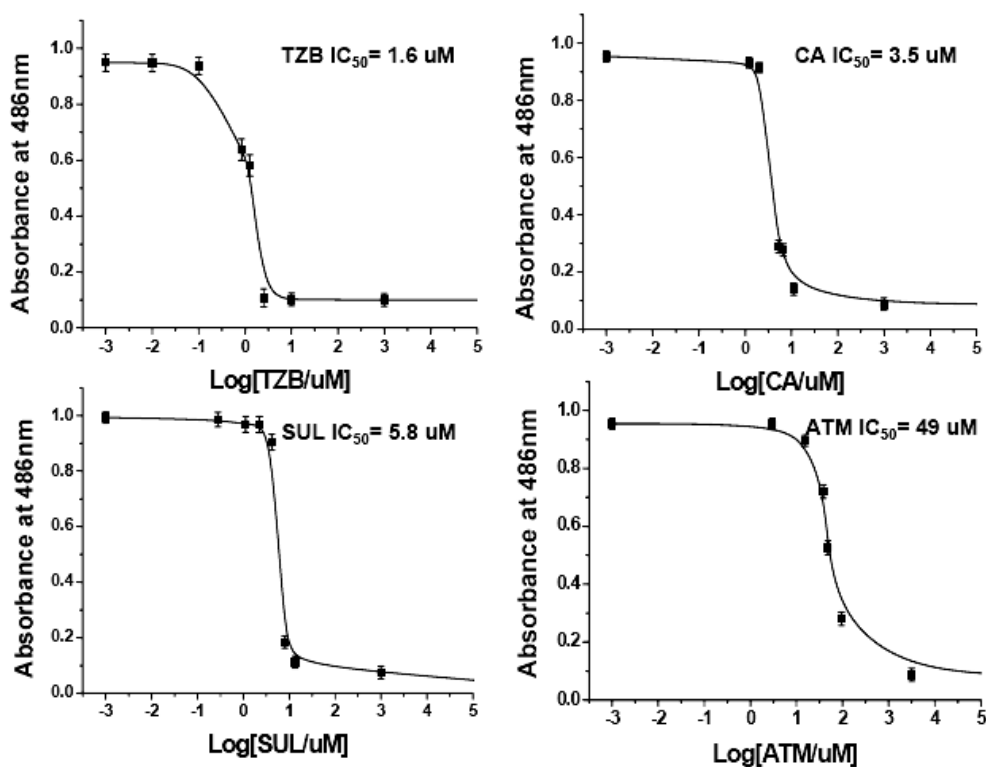


Figure S5. IC_{50} values of TZB, CA, SUL and ATM for transformed TEM-1 β -lactamase by using nitrocefin ($20 \mu\text{M}$).

10. Aggregation test with gold nanoparticles in bacterial strains.

All the bacterial strains were grown at 37°C in LB broth (Fischer). When the optical density (OD) at 600 nm reached 0.8, the suspension was chilled on ice for 5 min, 1 ml aliquots were taken into 1.5 mL vial, and bacteria were harvested by centrifugation at 3,000 rpm for 10 min. After centrifugation, supernatant was removed and cells were washed three times with 1 mL of Tris buffer. Then, bacterial cells were suspended in deionized water which contained substrates under the room temperature. The suspension was incubated for 20 minutes for further enzyme interactions. The bacterial solution was added into Au-NPs suspension to induce the aggregation of Au-NPs. After 40 min interaction, Au-NPs solution was separated from bacteria by centrifugation at 3,000 rpm for 3 min. And then the supernatant was applied for colorimetric image.

11. Colorimetric assay by using β -lactamase inhibitors and gold nanoparticles in bacteria.

Bacterial cells (β -lactam antibiotics resistant gene transformed *E. coli* B121 $\sim 10^8$ cfu/mL, TEM-1 *E. coli* (ATCC 35218) $\sim 10^9$ cfu/mL, *Bacillus cereus* (ATCC 13061) $\sim 8 \times 10^9$ cfu/mL, and clinical isolate

K. pneumoniae (ATCC 700603) $\sim 3 \times 10^8$ cfu/mL) were suspended in Tris buffer (pH 7.4). Inhibitor solutions were incubated with bacteria for further enzyme interactions. Then the pretreated bacterial suspension and substrate solution were added into Au-NPs suspension to induce the aggregation of Au-NPs. The final concentration of substrate and inhibitors were maintained at 8 μ M and 0.1 μ M, respectively. After 40 min interaction, Au-NPs solution was separated from bacteria by centrifugation at 3,000 rpm for 3 min. And then the supernatant was applied for colorimetric image.

12. Colorimetric assay by using β -lactamase inhibitors and nitrocefin in bacteria.

Bacterial cells (β -lactam antibiotics resistant gene transformed *E. coli* Bl21 $\sim 10^8$ cfu/mL, TEM-1 *E. coli* (ATCC 35218) $\sim 10^9$ cfu/mL, *Bacillus cereus* (ATCC 13061) $\sim 8 \times 10^9$ cfu/mL, and clinical isolate *K. pneumoniae* (ATCC 700603) $\sim 3 \times 10^8$ cfu/mL) were suspended in Tris buffer (pH 7.4). Inhibitor solutions were incubated with bacteria for further enzyme interactions. The pre-treated bacterial solution was added into nitrocefin solution. The final concentration of nitrocefin and inhibitors were maintained at 20 μ M and 3.0 μ M, respectively. After 15 min interaction, the solution was separated from bacteria by centrifugation at 3,000 rpm for 3 min. And then the supernatant was applied for colorimetric image.

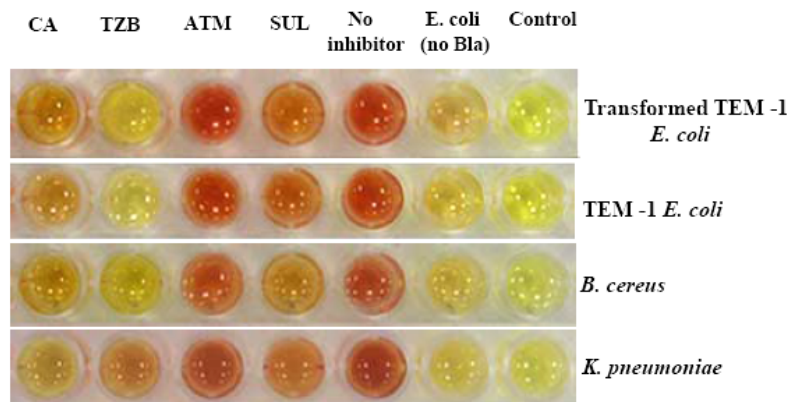


Figure S6. Nitrocefin based colorimetric assay for inhibitors screening. The concentration of inhibitors was maintained at 3.0 μ M, which was larger than that in Au-NPs assay.

13. Absorbance change ratio for β -lactamase inhibitors and gold nanoparticles or nitrocefin in bacteria.

β -lactam antibiotics resistant bacterial strains were suspended in Tris buffer (pH 7.4). Inhibitor solutions were incubated with bacteria for further enzyme interactions. The bacterial solution and substrate solution were added into Au-NPs or nitrocefin solution. The final concentration of substrate, nitrocefin and inhibitors were maintained at 8 μ M, 20 μ M and 0.1 μ M. After interaction, Au-NPs and

nitrocefin solution were separated from bacteria by centrifugation at 3,000 rpm for 3 min. And then the supernatants were used to measure the absorbance ratio at 650 nm/520 nm and 486 nm/390 nm, respectively.

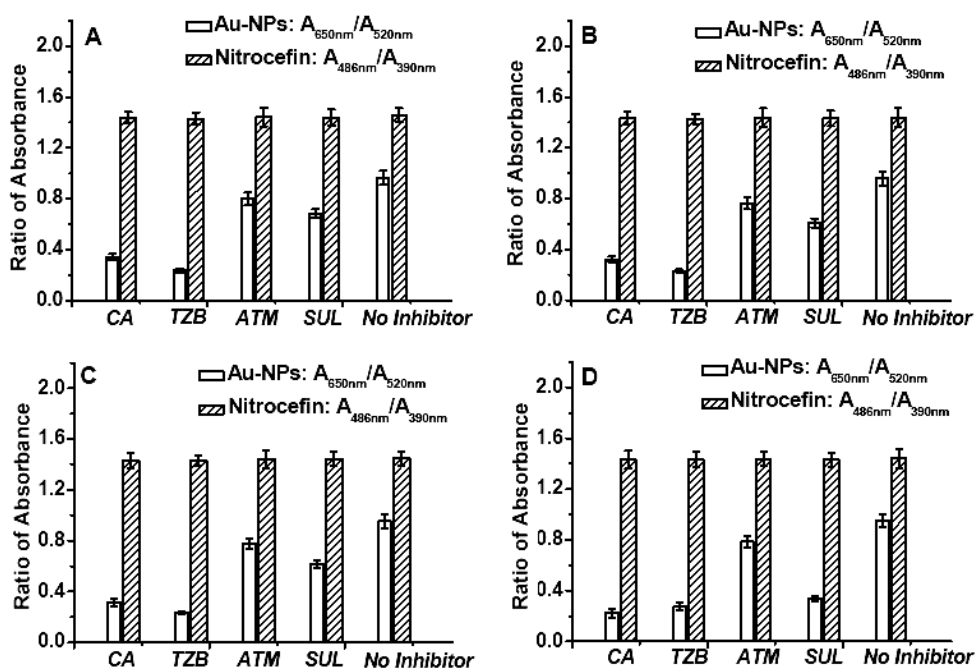


Figure S7. Absorbance change ratio of β -lactamase inhibition assay in the absence and presence of different inhibitors (inhibitors concentration: 0.1 μ M). (A: transformed TEM-1 *E.coli* B121, $\sim 10^8$ cfu/ml, B: TEM-1 *E.coli*, $\sim 10^9$ cfu/ml, C: *Bacillus cereus*, $\sim 8 \times 10^9$ cfu/ml, and D: *K. pneumoniae*, $\sim 3 \times 10^8$ cfu/ml)

References:

- 1). Turkevich, J.; Stevenson, P. C.; Hollier, J. *Discuss. Faraday Soc.* 1951, **11**, 55.
- 2). Choi, Y.; Ho, N. H.; Tung, C. H. *Angew Chem. Int. Ed.* 2007, **46**, 707