Supporting Information

Real-time activity monitoring of New Delhi metallo-β-lactamase-1 in living bacterial cells by UV-Vis spectroscopy

Ke-Wu Yang*[‡a], Yajun Zhou[‡a], Ying Ge[a], and Yuejuan Zhang[a]

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Materials and methods

1-1. Reagents

All antibiotics and inhibitors of EDTA and D-captopril used in this study were purchased from Sigma-Aldrich Corporation. The inhibitor azolylthioacetamide (Fig. S6) was synthesized as previously described method[1]. The six clinical bacterial strains were obtained from Fourth Military Medical University. *Escherichia coli* BL21 (DE3) cells were purchased from Wolsen Co. Ltd.

1-2. NDM-1 enzyme expression and purification

NDM-1 enzyme was overexpressed and purified based on the previously described[2]. NDM-1 plasmids were transformed into *E. coli* BL21(DE3) cells, and the transformation mixtures were spread into lysogeny broth (LB) plates containing 25 μg/mL kanamycin. A single colony was transferred into 50 mL of LB containing 25 μg/mL kanamycin, and the culture was allowed to shake overnight at 37 °C. The overnight culture (10 mL) was transferred into 4 × 1 L of LB containing 25 μg/mL kanamycin. The resulting culture was grown at 37 °C with shaking at 220 rpm until an OD_{600} of 0.6−0.8 was reached. Protein production was induced by making the cultures 1 mM in IPTG. The cells were allowed to shake for 3 h at 37 °C, and then the cells were harvested by centrifugation for 30 min at 8000 rpm and 4 °C. The pellets were resuspended in 25 mL of 30 mM Tris (pH 8.0) containing 500 mM NaCl. The cells were lysed by Ultrasonic. The insoluble components were removed by centrifugation for 30 min at 18000 rpm. The supernatant was dialyzed versus 30 mM Tris (pH 8.0) containing 100 μM $ZnCl_2$ for 36 h. After centrifugation for an additional 25 min at 18000 rpm to remove the insoluble components, the cell lysate was loaded onto a Q-sepharose column that had been equilibrated with 30 mM Tris (pH 8.0) containing 100uM $ZnCl_2$.Proteins were eluted using a linear gradient from 30 mM Tris (pH 8.0) containing 100 μM $ZnCl_2$ to 30 mM Tris (pH 8.0) containing 500 mM NaCl. Column fractions containing the crude NDM-1 protein were identified using a 15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel, and fractions containing NDM-1 were collected and concentrated to 2−3 mL using an Amicon ultrafiltration concentrator equipped with an YM-10 membrane. The concentration of crude NDM-1 protein went through a G75 column. Proteins were eluted with 30mM Tris(pH 7.5) containing 200 mM NaCl. The purified NDM-1 protein was identified by SDS−PAGE from column fractions.

1-3. *E. coli* cells preparations for in vivo UV-Vis studies

We transformed the plasmids *pET26b* (with NDM-1gene or not) and the plasmid *PMSZ02-CcrA* to *Escherichia coli* cells, respectively. The kanamycin resistance gene was contained in the plasmids. *E. coli* cells were inoculated into 5 mL lysogeny broth, LB, media in the presence of 25 μg/mL kanamycin and were grown with shaking (150 rpm) at 37 ºC until cells reached OD_{600}= 0.6. At that time, 100 μM IPTG was added for metallo-beta-lactamase induction and cells were grown for 2 hours at 37 ºC with shaking (150 rpm). Cell cultures were centrifuged at 4000 rpm for 10

min at 4 ºC, the supernatant was discarded and the cell pellets were washed thoroughly by re-suspending them in 1 mL buffer (50 mM sodium phosphate, pH 7.0), and were then pelleted again by centrifugation (4000 rpm, for 10 min at 4 ºC). This process was repeated 3 times and finally cells were re-suspended in buffer to OD_{600} = 0.2 for UV-Vis studies. Different concentrations of each inhibitor and/or 100 μM of cefazolin were added for estimation of the IC_{50} values. Supernatants from the cell suspensions were collected by centrifugation (10000 rpm for 10 min at 4 ºC) and then filtered through 0.22 μm filters.

1-4. Plating colony tests for NDM-1 *E. coli* cells

We obtained serial 10, 10^2, 10^3, 10^4, 10^5 and 10^6–fold dilutions from stock suspensions of NDM-1 *E. coli* cells (OD_{600} = 0.2). Then, we inoculated an LB−agar plate containing 25 μg/mL kanamycin with 10 μL drops of each dilution. The drops were allowed to dry on the plate and the plate was incubated at 37 °C overnight.

1-5. UV-Vis measurements and analyses

All spectra were acquired on Agilent 8453 spectrometer. In the mode of standard, different concentrations of each inhibitor was added into several cuvettes with same stock suspensions of NDM-1 *E. coli* (OD_{600} = 0.2) as parallel experiment groups. After the same concentration of substrate was added into every group, the UV-Vis spectra of the substrate cefazolin in the cuvettes were acquired with 3 scans per 10 min until the characteristic absorption peak didn't change and the temperature was kept constant at 25 ºC. It is easily to obtain the concentrations of the substrate by the UV absorbance.

In the mode of kinetics, the suspensions bacteria cells mixed with the substrate were set as background blank. The spectrometer was set up for monitoring at the characteristic absorption peak of the substrate, such as cefazolin is 265 nm, to acquire spectra automatically 1 scan per 10 min in 3h.

1-6. IC_{50} measurements and analyses

We calculated the percentage of the inhibition seen 70 minutes, when the substrate in the absence of inhibitor was almost completely hydrolyzed, after the initiation of the reaction using the equation below which was according to the method introduced by Dalvit et al[3]. (Equation 1):

$$\% \text{ inhibition} = 100 \times [1 - ([S_T] - [S_I])/ ([S_T] - [S_O])] \quad \text{[Equation 1]}$$

Where $[S_T]$ is the intial concentration of the substrate and $[S_O]/[S_I]$ is the real-time concentration of the substrate in the absence or presence of inhibitor, respectively.

IC_{50} can be obtained by fitting the data of % inhibition vs. inhibitor concentration to Equation 2:

$$\% \text{ inhibition} = 100 \times [1 - 1/ (1 + ([I]/IC_{50})^n)] \quad \text{[Equation 2]}$$

Where [I] is the concentration of the inhibitor and n is the cooperativity factor.

Supporting figures

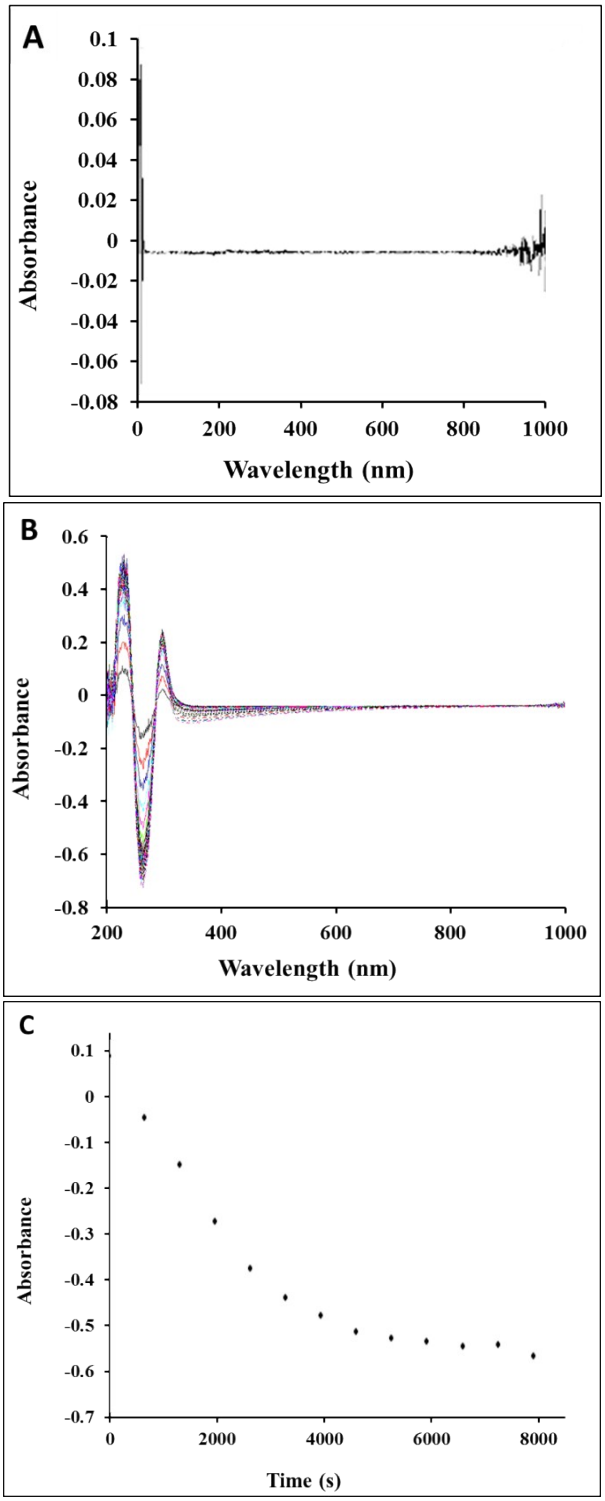

Fig. S1 UV-Vis spectrum of 100 µM cefazolin in the presence of NDM-1 *E. coli* cell suspension (OD_{600} = 0.2) in the mode of kinetics. Setting the background signals from the cells and sample preparation as blank (A); the cefazolin was being hydrolyzed by NDM-1 *E. coli* cells (B); and monitoring the absorbance of the cefazolin at 265 nm (C).

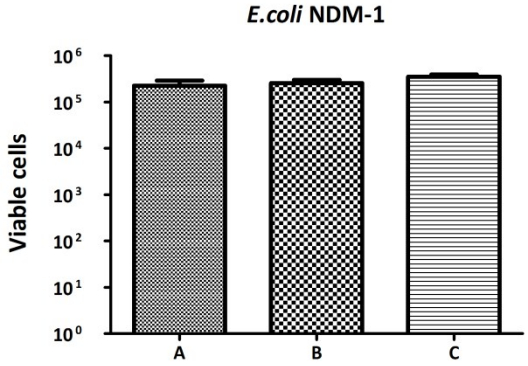

Fig. S2 The average number of colonies in triplicate of NDM-1 *E. coli* cell without the treatment of cefazolin (A) and with the addition of 100 μM cefazolin before (B) and after (C) three-hour UV-Vis experiments, respectively.

Plating colony tests were performed to examine the viability of NDM-1 *E. coli* cell before and after UV-Vis experiments. All NDM-1 *E. coli* cells from the same batch were serilly diluted and plated on LB/kanamycin plates. NDM-1 *E. coli* cells without the treatment of cefazolin were plated on plate A. Cells with the addition of 100 μM cefazolin before and after three-hour UV-Vis experiments were plated on plate B and C, respectively. The section with 10^5 fold dilution is used for the study of cell viability. The clolonies grown on the plates were counted. The average number of colonies in triplicate was extracted to give the column graph A, B and C showed in Fig S2. It showed that the cells were alive during the UV-Vis experiments and there was no significant difference of cell viability before and after the experiments ($P>0.05$).

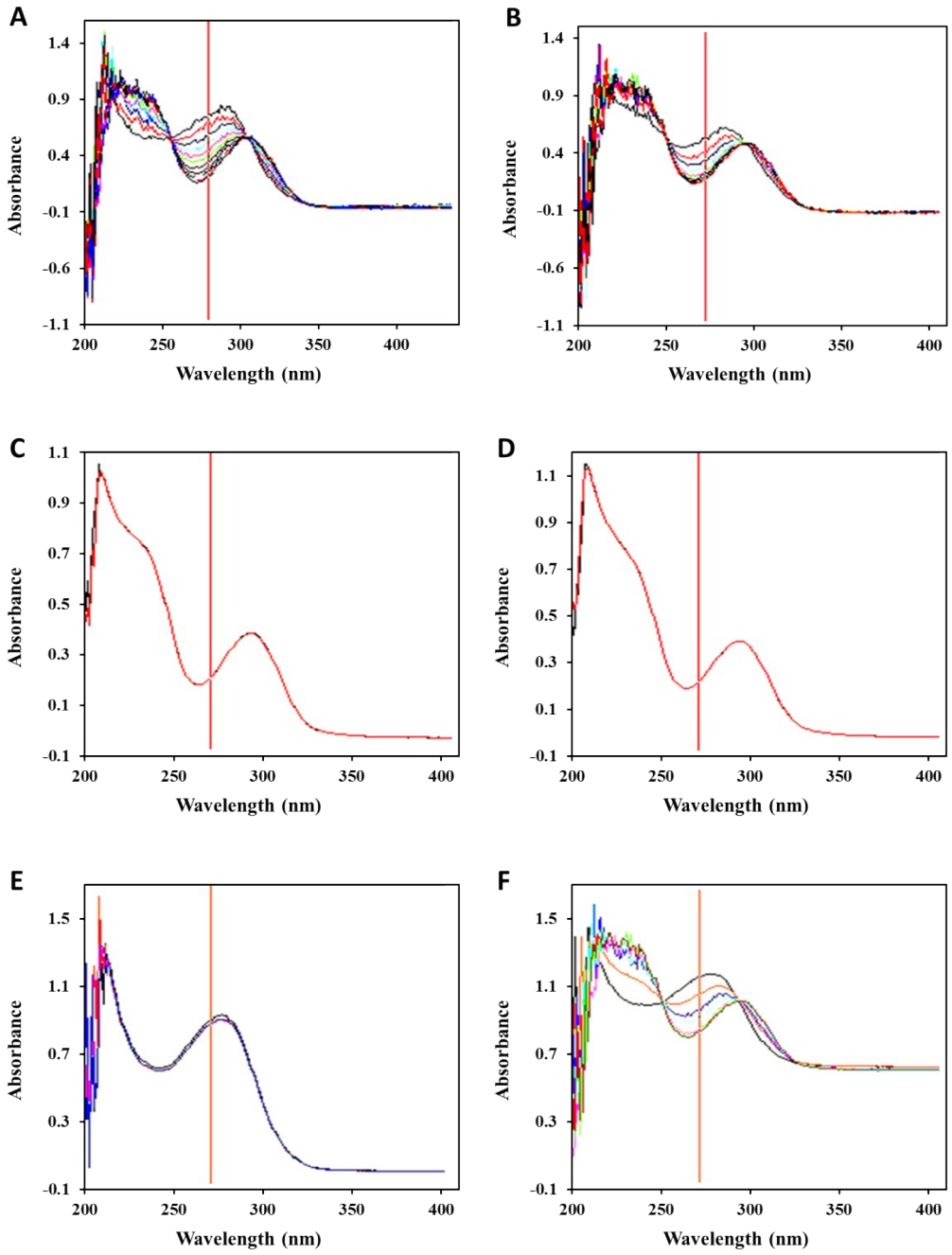

Fig. S3 Monitoring of the cefazolin hydrolysis in the presence of NDM-1 *E. coli* cells and CcrA *E. coli* cells, respectively. UV spectra of NDM-1 (A, sample A) and CcrA (B, sample B) *E. coli* cells (OD_{600} = 0.2) with the initial addition of 100 µM cefazolin; the sample A and B were incubated for 1 hour, centrifuged, and the resulting supernatant C and D to offer the UV spectrum C and D, respectively; the supernatant C and D were added another 100 µM fresh cefazolin to offer the UV spectrum E and F, respectively. All samples were prepared with 50 mM sodium phosphate buffer, pH 7.0.

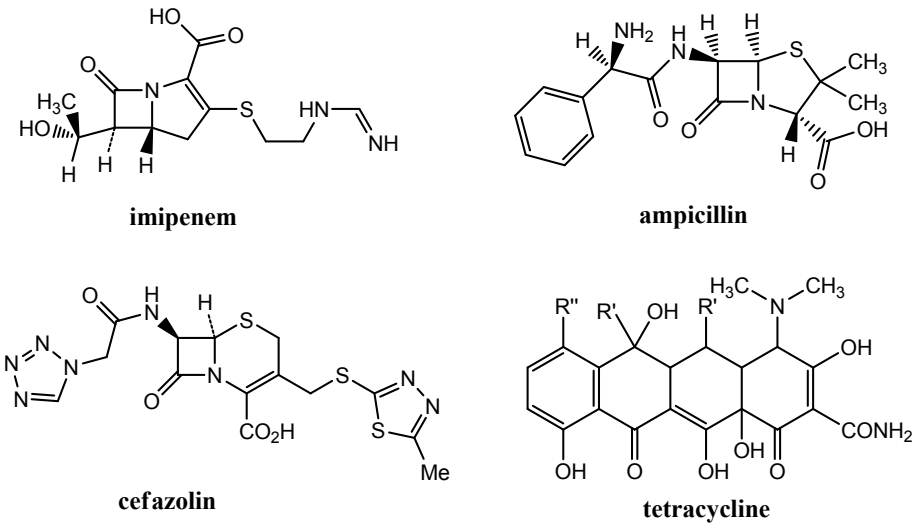

Fig. S4 Chemical Structures of antibiotics selected for stability test.

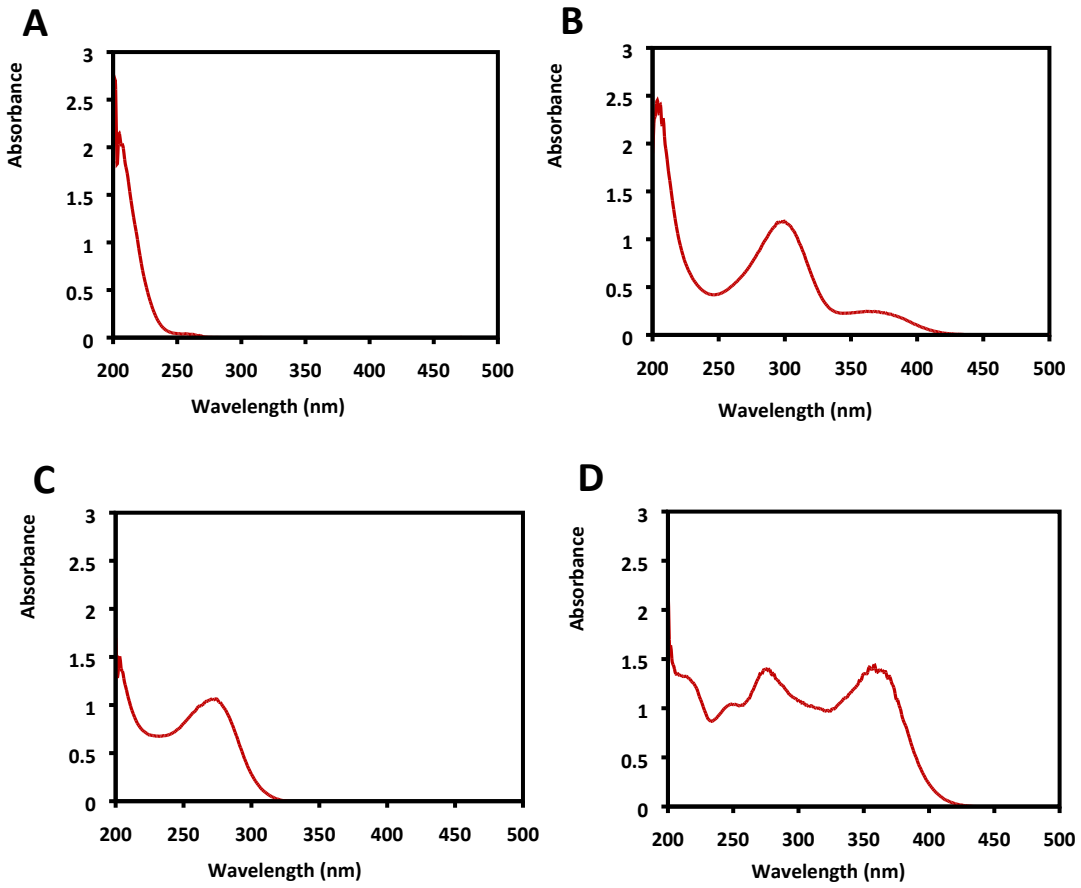

Fig. S5 UV-Vis spectra of ampicillin (A), imipenem (B), cefazolin (C) and tetracycline (D) in 50 mM sodium phosphate buffer, pH 7.0.

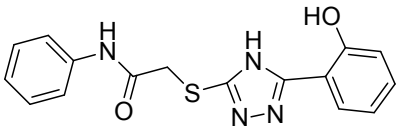

Fig. S6 Chemical Structure of the inhibitor azolylthioacetamide.

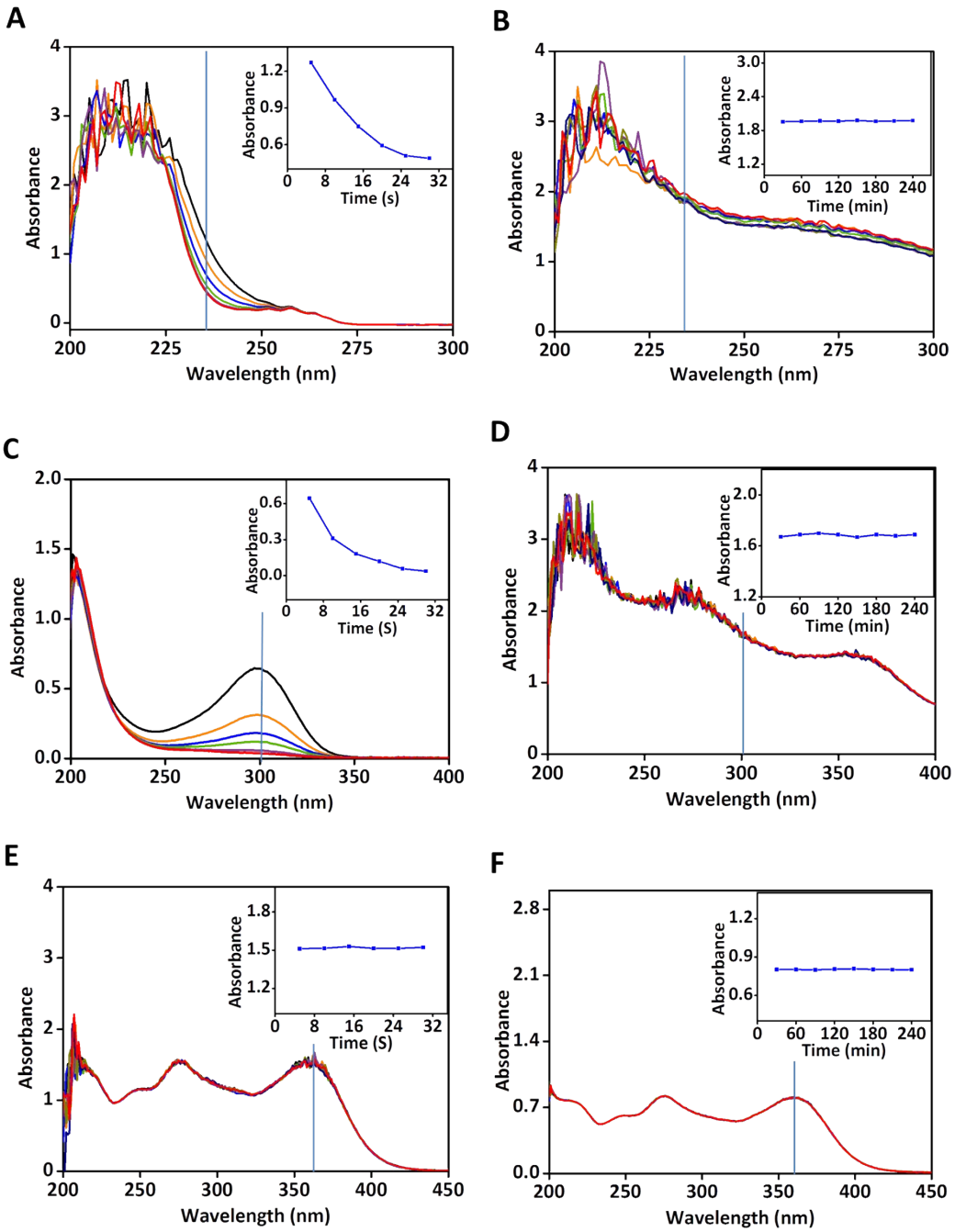

Fig. S7 UV-Vis spectra of hydrolysis of three antibiotics with the purified NDM-1 enzyme (A: ampicillin; C: imipenem; E: tetracycline) and with the *E. coli* cells without NDM-1 (B: ampicillin; D: imipenem; F: tetracycline). The concentration of the antibiotic is 100 μM, in a 50 mM sodium phosphate buffer, pH 7.0.

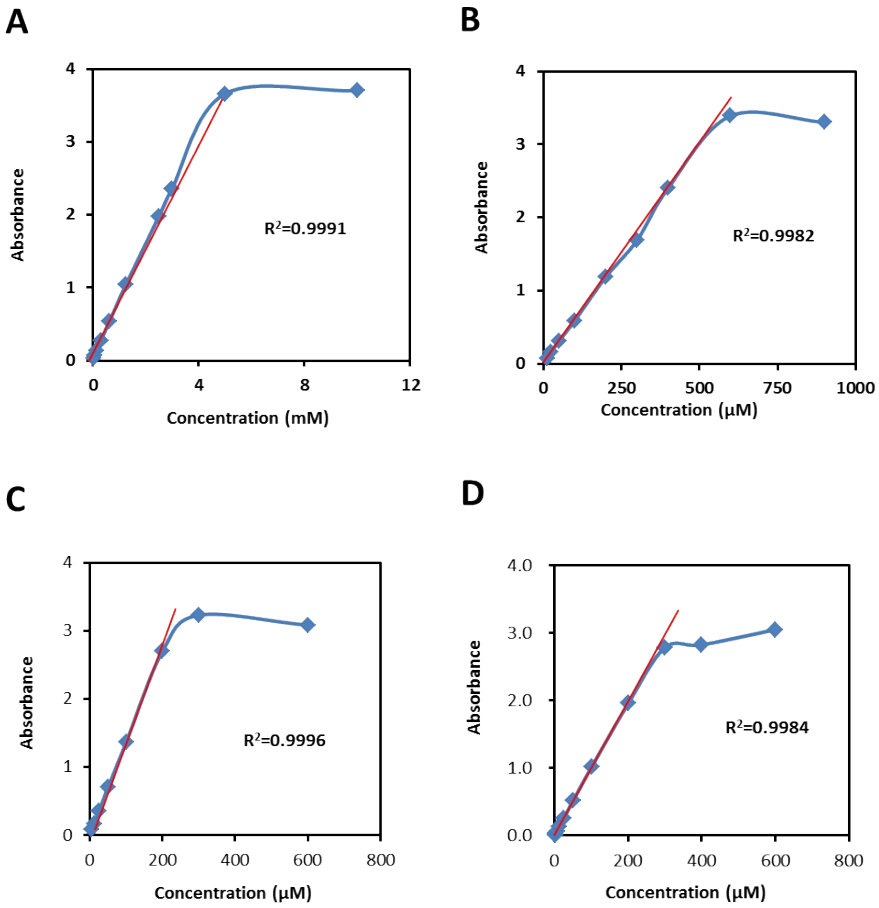

Fig. S8 The absorbance changes with concentrations of the antibiotics tested. The calibration curves showing the linearity relation for the antibiotics ampicillin (A), imipenem (B), tetracycline (C), and cefazolin (D). All samples were prepared with 50 mM sodium phosphate buffer, pH 7.0.

References

[1] Y.-L. Zhang, K.-W. Yang, Y.-J. Zhou, A. E. LaCuran, P. Oelschlaeger, M. Crowder, *ChemMedChem.,* 2014, **9**, 2445.

[2] H. Yang, M. Aitha, A. Hetrick, T. Richmond, D. Tierney, M. Crowder, *Biochemistry,* 2012, ***51***, 3839.

[3] C. Dalvit, E. Ardini, G. P. Fogliatto, N. Mongelli, M. Veronesi, *Drug Discov. Today,* 2004, ***9***, 595.