Supporting Information

Real-time monitoring and inhibition of carbapenemase activity in live bacterial cells: application to screening of β-lactamase inhibitors

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Materials and methods

1-1. Reagents

All antibiotics, EDTA, D-captopril and clavulanic acid used in this study were purchased from Sigma-Aldrich Corporation. The inhibitors rhodanine and ebselen (Fig. S6) were synthesized as previously described method[1,2]. The clinical strains *P. aeruginosa*, *K. pneumoniae*, Methicillin-resistant *S. aureus* (MRSA), *E. coli* harboring extended-spectrum β-lactamases (ESBL-*E. coli*), *E. coli* cells producing NDM-1 (EC07) and *S. aureus* were obtained from the Air Force Military Medical University, Xi'an, China. NDM-*K. pneumoniae* and KPC-*K. pneumoniae* were obtained from PLA, Southern Theater General Hospital, Guangzhou, China, and *E. coli* BL21 (DE3) cells were purchased from Wolsen Co. Ltd.

1-2. Expression of VIM-2

A gene encoding VIM-2 (GenBank code: EU912537.1) with *Escherichia coli* (*E. coli*) codon usage was synthesized by GENERAY. The bla_{VIM-2} gene was first amplified with the forward primer 5-TCCATATGTTCAAACTTTTGAGT-3′ and the reverse primer 5′-TCGAGGATCCTGCTACTCAACGACTG-3′. Next, the PCR product was digested with NdeI and XhoI restriction endonucleases and cloned into the vector pET-24a. Subsequently, the plasmid was transformed into *E. coli* strain BL21-Gold (DE3).

The protein sequence of the VIM-2 used in the enzymatic assay is listed below:
MFKLLSKLLVYLTASIMAIASPLAFSVDSSGEYPTVSEIPVGEVRLYQIADGVWSHIAT
QSFDGAVYPSNGLIVRDGDELLLIDTAWGAKNTAALLAEIEKQIGLPVTRAVSTHFHD
DRVGGVDVLRAAGVATYASPSTRRLAEVEGNEIPTHSLEGLSSSGDAVRFGPVELFY
PGAAHSTDNLVVYVPSASVLYGGCAIYELSRTSAGNVADADLAEWPTSIERIQQHYP
EAQFVIPGHGLPGGLDLLKHTTNVVKAHTNRSVVE

VIM-2 enzyme was overexpressed and purified as previously described[3]. VIM-2 plasmids were used to transform *E. coli* BL21 (DE3), 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 ℃. The overnight culture (10 mL) was transferred into 4 × 1 L of LB containing 25 μg/mL kanamycin. The resulting culture grown at 37 ℃ with shaking at 150

rpm until an OD_{600} of 0.6−0.8 was reached. Protein production was induced by making the cultures 0.5 mM in IPTG. The cells were allowed to shake for 4 h at 37 ℃, and then the cells were harvested by centrifugation for 20 min at 6000 rpm and 4 ℃. The pellets were resuspended in 25 mL of 30 mM Tris (pH 7.6). The cells were lysed by Ultrasonic. The insoluble components were removed by centrifugation for 20min at 12000 rpm. The supernatant was dialyzed versus 30 mM Tris (pH 7.6) for 24 h. After centrifugation for an additional 20 min at 12000 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 7.6). Proteins were eluted using a linear gradient from 30 mM Tris (pH 7.6) to 30 mM Tris (pH 7.6) containing 500 mM NaCl. The purified VIM-2 protein was identified by SDS−PAGE from column fractions. The purified VIM-2 protein was identified by SDS−PAGE from column fractions (Fig. S1).

1-3. Preparation of *E. coli* cells

The plasmids pET24a-VIM-2 were used to transform *E. coli* BL21 (DE3). *E. coli* cells inoculated into 5 mL lysogeny broth (LB) media in the presence of 25 μg/mL kanamycin grown with shaking (150 rpm) at 37 ℃ until cells reached OD_{600} = 0.5-0.6, and then, The production of the VIM-2 was induced by making the culture 100 μM in IPTG, and the cells grown for 2 hours at 37 ℃ with shaking (150 rpm). Cell cultures were centrifuged at 4000 rpm for 10 min at 4 ℃, 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 ℃). This process was repeated 3 times and finally cells were re-suspended in same buffer to OD_{600} = 0.15 for UV-Vis studies. Different concentrations of each inhibitor and 170 μM faropenem 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 ℃) and then filtered through 0.22 μm filters.

1-4. Plating colony tests

We obtained 10^5 fold dilutions from stock suspensions of VIM-2 *E. coli* and *E. coli* cells (OD_{600} = 0.15). Then, we inoculated LB-agar (with and without kanamycin) plates with 10 μL drops of each dilution for the average number of colonies in triplicate. The drops were allowed to dry on the plate and the plate was incubated at 37 ℃ overnight.

1-5. Measurements and analyses of IC_{50}

We calculated the percentage of the inhibition seen 110-330 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[4]. (Equation 1):

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

Where $[S_T]$ is the initial 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.

1-6. Measurements and analyses of molar absorption coefficients

The molar absorption coefficient, ε (λ) (in M^{-1} cm^{-1}), expresses the ability of a compound to absorb radiation at a specific wavelengthλ. Therefore, the molar absorption coefficients of antibiotics were measured according to the Beer-Lambert law, in the UV region of the electromagnetic spectrum.[5]

(Equation 3):

$$A (\lambda) = \varepsilon (\lambda) \cdot c \cdot d.$$

where:

A (λ) in Equation 3: absorbance at wavelength λ; ε (λ): the molar absorption coefficients of the antibiotics at wavelength λ (M^{-1} cm^{-1}); c: the concentration in solution, and d: the optical path length of the cuvette[6].

Supporting figures

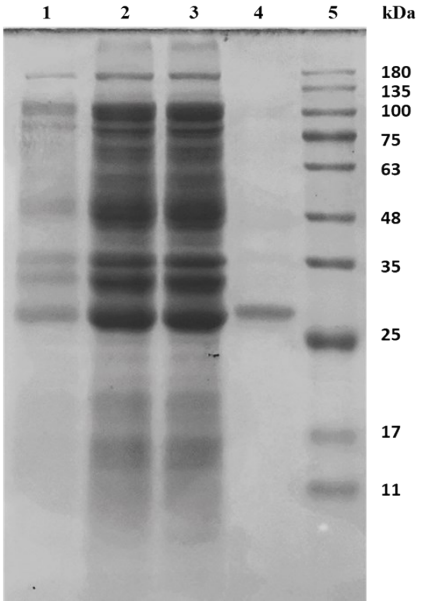


Fig. S1 SDS-PAGE of VIM-2 purification. Lane 1: total cell lysate of *E. coli* BL21 transformed with expression vector encoding the VIM-2 protein before IPTG induction. Lane 2: total cell lysate of *E. coli* BL21 transformed with expression vector encoding the VIM-2 protein after 0.5 mM IPTG induction. Lane 3: supernatant crude protein after crushing and centrifugation. Lane 4: purified VIM-2 protein eluted from Q-Sepharose column. Lane 5: protein molecular weight marker.

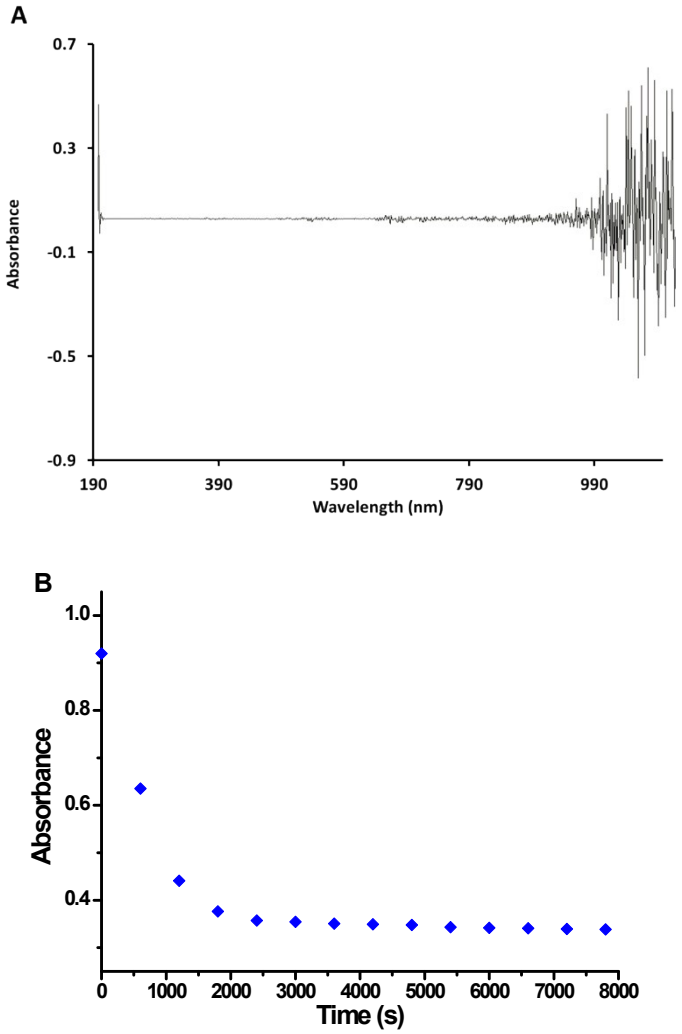


Fig. S2 UV-Vis spectrum of 170 μM faropenem in the presence of VIM-2 *E. coli* cell suspension (OD_{600} = 0.15) in the mode of kinetics. Setting the background signals from the cells and sample preparation as blank (A); the faropenem was being hydrolyzed (B)

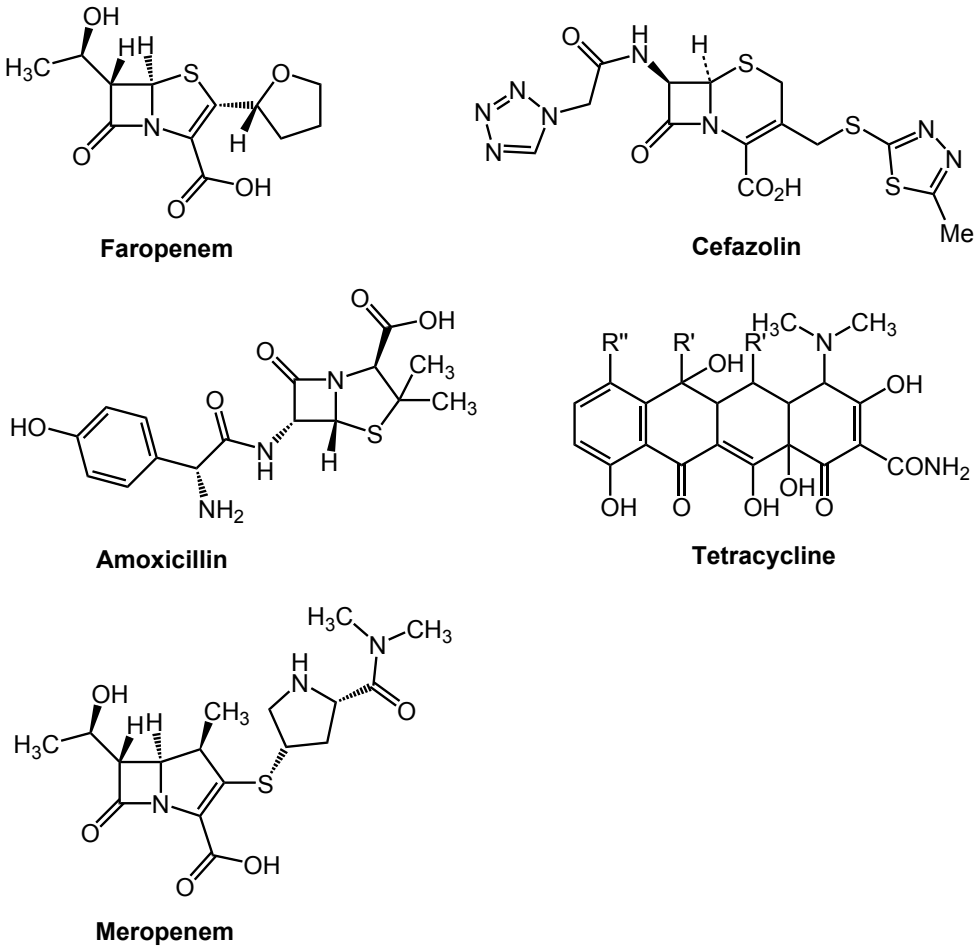
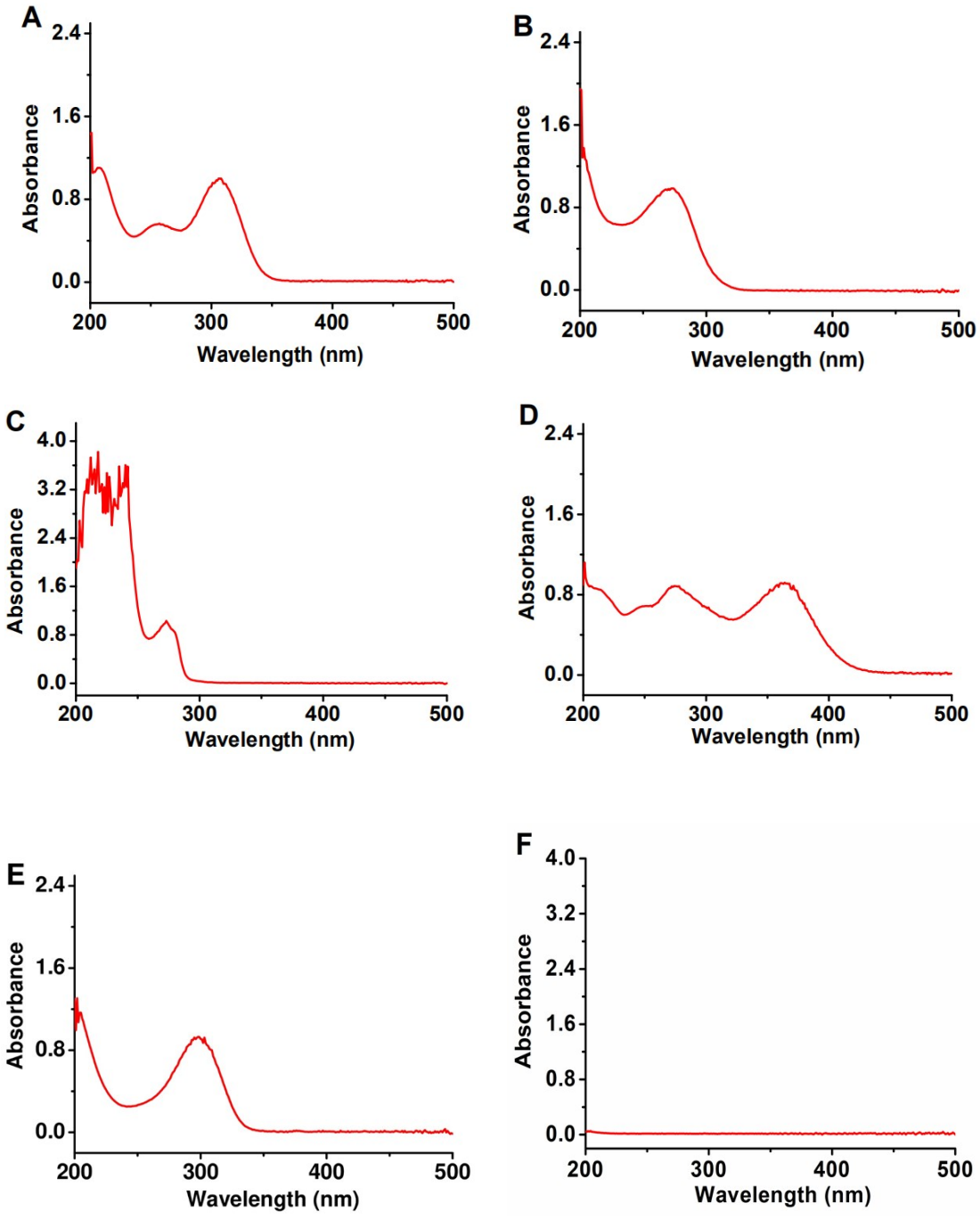


Fig. S3 Structures of antibiotics faropenem, cefazolin, amoxicillin, tetracycline and meropenem.


A
Absorbance
2.4
1.6
0.8
0.0
200
300
400
500
Wavelength (nm)
B
Absorbance
2.4
1.6
0.8
0.0
200
300
400
500
Wavelength (nm)
C
Absorbance
4.0
3.2
2.4
1.6
0.8
0.0
200
300
400
500
Wavelength (nm)
D
Absorbance
2.4
1.6
0.8
0.0
200
300
400
500
Wavelength (nm)
E
Absorbance
2.4
1.6
0.8
0.0
200
300
400
500
Wavelength (nm)
F
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200
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400
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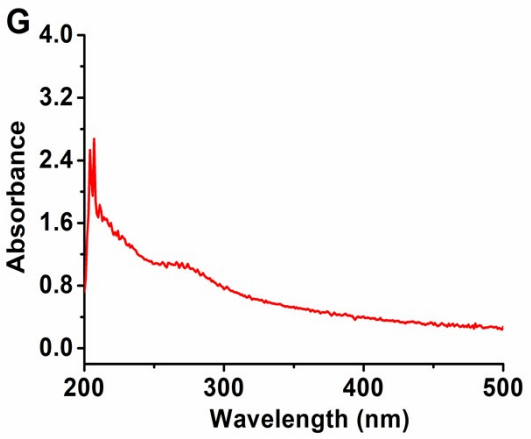
Wavelength (nm)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Wavelength (nm)
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300
400
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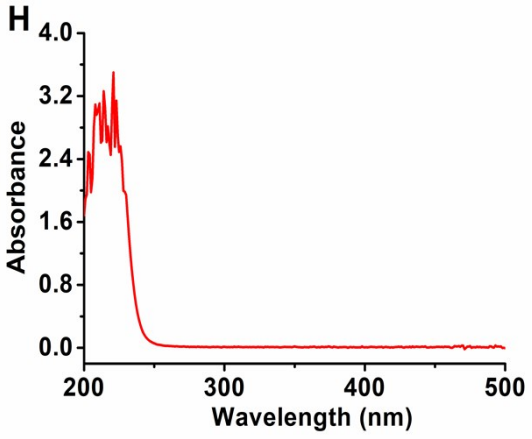
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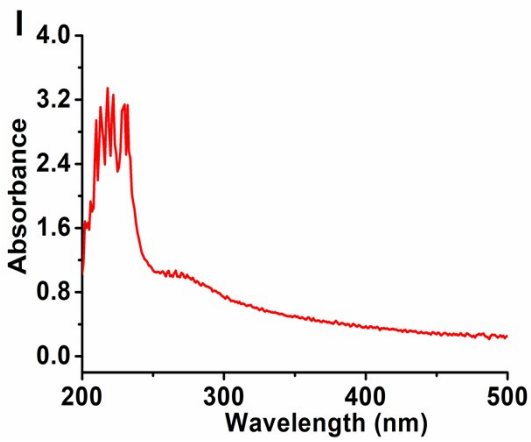
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Wavelength (nm)
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1.6
0.8
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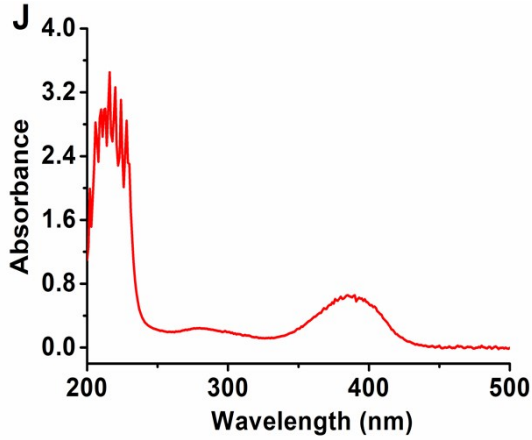
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Wavelength (nm)
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0.8
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200
300
400
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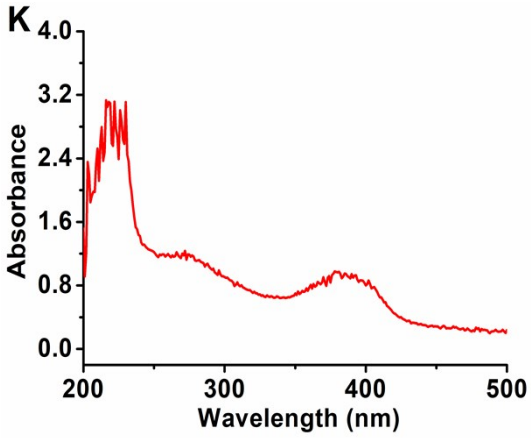
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Absorbance
Wavelength (nm)
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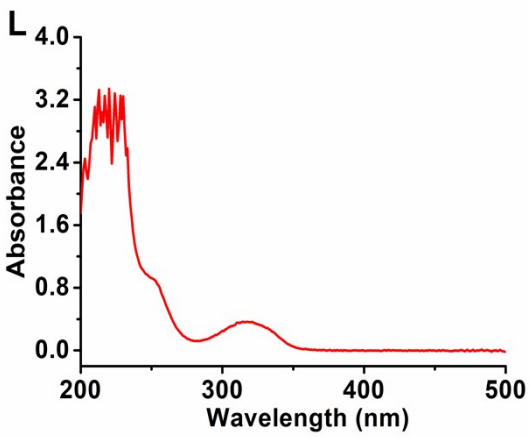
1.6
0.8
0.0
200
300
400
500


L
Absorbance
Wavelength (nm)
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1.6
0.8
0.0
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300
400
500

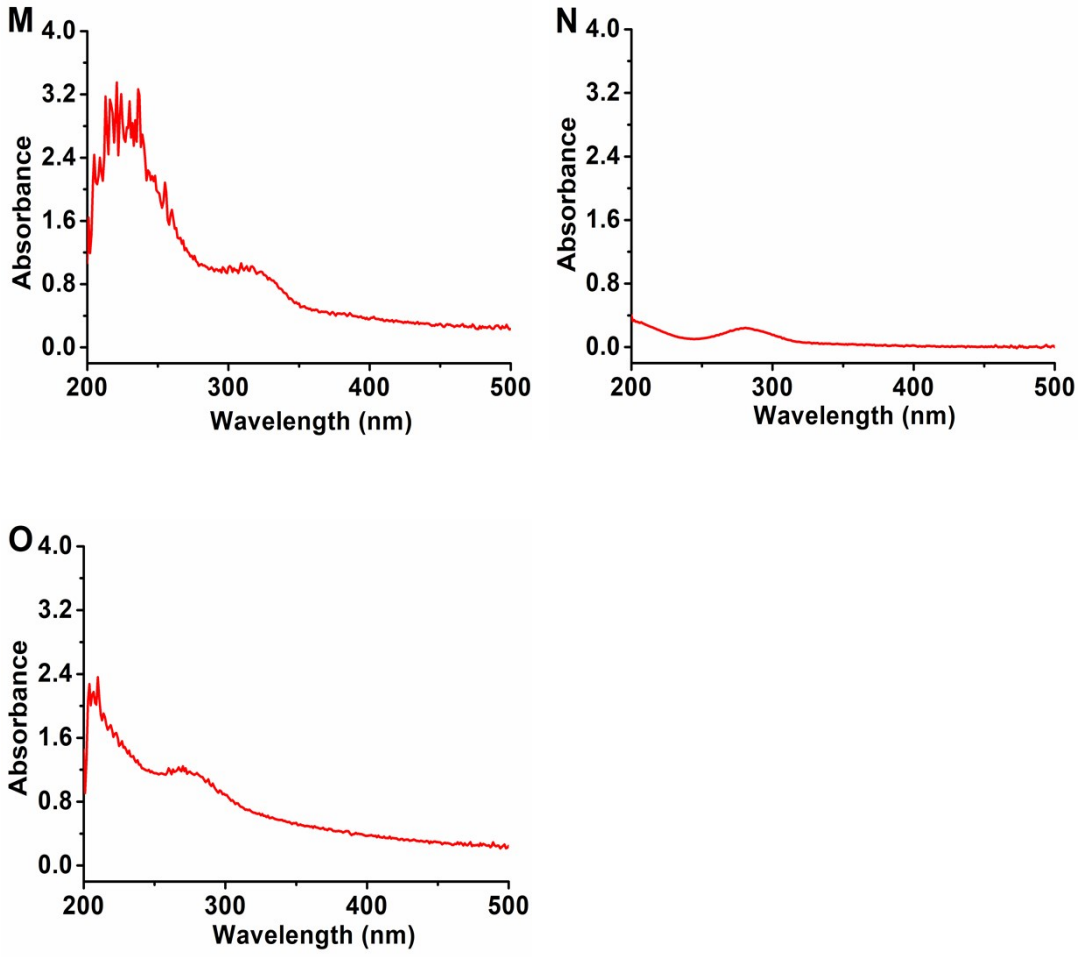


Fig. S4 UV-Vis spectra of faropenem (A), cefazolin (B), amoxicillin (C), tetracycline (D), and meropenem (E), EDTA (F), EDTA with *E. coli* cells producing VIM-2 (G), D-captopril (H), D-captopril with *E. coli* cells producing VIM-2 (I), rhodanine (J), rhodanine with *E. coli* cells producing VIM-2 (K), ebselen (L), ebselen with *K. pneumoniae* producing NDMs (M), clavulanic acid (N), clavulanic acid with *K. pneumoniae* producing KPC (O). All of the cell pellets were resuspended to OD_{600} = 0.15. For the monitoring of antibiotic hydrolysis, before the monitoring, the initial absorbance of each inhibitor mixed with bacterial cells was set as blank (base line). In this way, the influence of absorbance of inhibitor and bacterial cells on the monitoring was deducted.

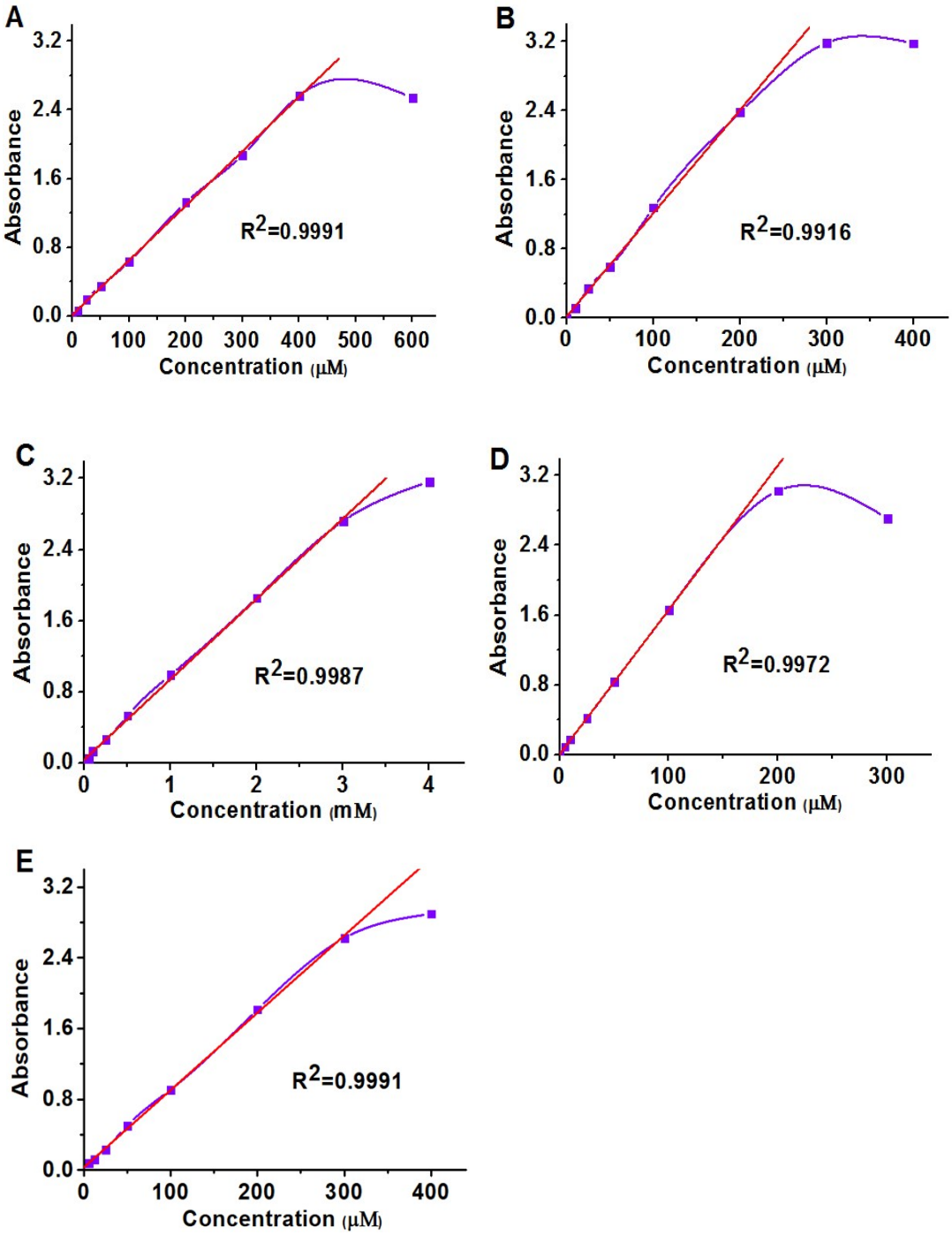
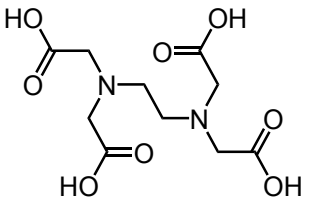
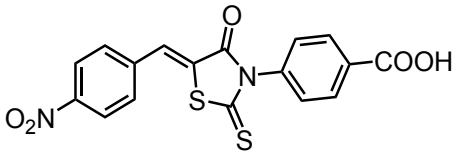


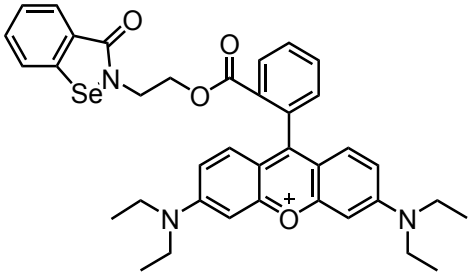
Fig. S5 The absorbance changes with concentrations of the antibiotics tested. The calibration curves showing the linearity relation for the antibiotics faropenem (A), cefazolin (B), amoxicillin (C), tetracycline (D), and meropenem (E). The molar extinction coefficient (ε) of faropenem, cefazolin, amoxicillin, tetracycline, and meropenem at 307, 265, 254, 360 and 300 nm was measured according to the Beer-Lambert law to be 7040, 12690, 987, 16580 and 10400 $M^{-1}cm^{-1}$, respectively. The determined data (987 and 10400 $M^{-1}cm^{-1}$) of amoxicillin and meropenem are slightly less than the data (1006 and 10700 $M^{-1}cm^{-1}$) that literatures reported[7, 5]. All samples were prepared with 50 mM phosphate buffer, pH 7.0.



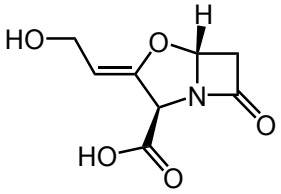
EDTA



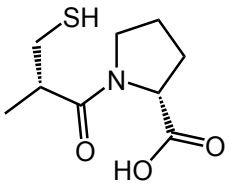
Rhodanine



Ebselen



Clavulanic acid



D-captopril

Fig. S6 Structures of EDTA, D-captopril, rhodanine, ebselen, and clavulanic acid.

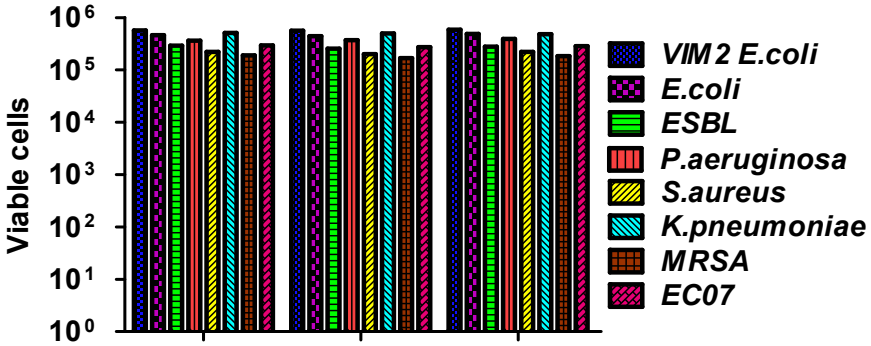


Fig. S7 Plating colony tests to examine the viability of all the cells before and after UV-Vis experiments and gained the average number of colonies in triplicate. All cells in these tests were from the same batch. The fold of dilution is labeled on each section of the LB (with and without kanamycin) plates. The cells without the treatment of faropenem were plated on plate A. Cells with the addition of 170 μM faropenem before and after three-hour UV-Vis experiments were plated on plate B and C, respectively. The section labeled with 10^5 fold dilution is used for the study of cell viability. This data demonstrate that the cells were alive during the UV-Vis experiments and there is little difference of cell viability before and after the experiments. ($P > 0.15$. No significant difference).

Supporting table

Table S1. MICs of faropenem against BL21 and *S. aureus.*

Bacteria	MIC (μg/mL)	Susceptibility
BL21	2.0	sensitive
S. aureus	0.5	sensitive

References

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2. C. Chen, Y. Xiang, K. W. Yang, Y. Zhang, W. M. Wang, J. P. Su*,* Y. Ge, Y. Liu*, Chem. Commun (Camb).,* 2018, **54**, 4802-4805.
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