

Colorimetric screening of bacterial enzyme activity and inhibition based on the aggregation of gold nanoparticles†

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A simple and novel gold nanoparticle (Au-NPs) based colorimetric method has been developed for efficient screening of class A β -lactamase (Bla) activity and inhibitors *in vitro* and in bacterial strains.

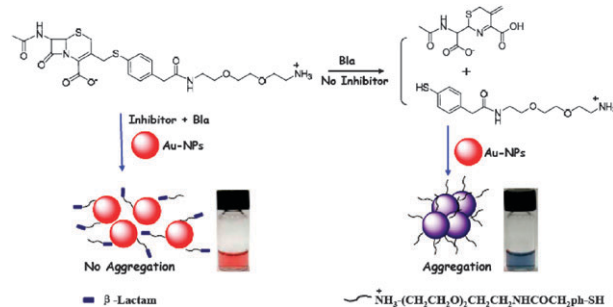
Since the antibiotic properties of penicillin were first discovered in the beginning of the last century, β -lactam antibiotics have been well developed as miracle drugs in the treatment of bacterial infections in clinics. However, increased bacterial resistance to β -lactam antibiotics has been extensively documented and has now become a serious public health threat. The most common mechanism for β -lactam antibiotic resistance is the production of a family of bacterial enzyme: β -lactamases (Blas),¹ a type of enzymes secreted both by gram-positive and gram-negative bacteria. Among the different members of the Bla family, class A Bla is one of the most prevalent plasmid-encoded or chromosomally encoded bacterial enzymes for mediating β -lactam resistance through the efficient hydrolysis of the β -lactam ring in penicillins and cephalosporins.² One practical method utilized in clinics to improve the antibiotic efficacy and combat bacterial resistance caused by these enzymes is to combine the β -lactam antibiotics with Bla inhibitors.^{2,3} As such, development of molecules that inhibit the Bla, and similarly, the specific methods for the high-throughput screening of efficient Bla inhibitors have become extremely crucial in clinical settings. Although some commonly used chromogenic (*e.g.*, nitrocefin) or fluorescent (*e.g.* protein fusion and genotyping based on polymerase chain reaction (PCR)) assays are able to successfully perform such tasks.⁴ Other methods based on hydrogels, phage display, and dynamic light scattering have also been developed to identify the Bla inhibitors *in vitro*.⁵ A simple and novel method for the efficient screening of Bla inhibitors is still necessary since most of the current methods have high reliance on specific instruments, require tedious sample pre-treatment, or do not provide efficient measurements in real-time.

Recently, metal nanoparticles (NPs) such as silver or gold have received much attention in bio-nanotechnology because of their unique optical and physical properties.⁶ Considerable studies have shown that silver or gold NPs can be extensively

exploited to monitor specific biomolecular recognition, such as in DNA hybridization or aptamer identification,⁷ sugar or metal ions tests,⁸ and toxin, amino acids or enzyme activities detection.⁹ Herein, we present a simple and specific gold NPs-based colorimetric method, which enables for simultaneous determination of the bacterial enzyme activity in the absence and presence of enzyme inhibitors *in vitro* and in bacterial strains. This new method could serve as an alternative platform for efficient screening of the bacterial enzyme inhibitors with the naked eyes or a simpler 96-well microtiter plate.

Scheme 1 outlines the principal design of a monomeric β -lactam substrate for Bla activity and inhibition screening. A flexible 2-(4-mercaptophenyl) acetic acid coupled 1,2-bis(2-aminoethoxy) ethane linker is connected to the 3'-position of cephalosporin through iodo-thiol substitution. As an excellent leaving group, the thiol group facilitates the release of the fragment on the β -lactam ring upon enzyme hydrolysis. The free thiol terminal and positively charged amino groups in the released fragment lead to the aggregation of Au-NPs based on the Au-S bond and electrostatic interaction between the charged amino group and the citrate ions on the surface of gold nanoparticles,^{9c} thus demonstrating the significant color change from red to blue. This red-shifting aggregate can be used as a colorimetric sensor to identify Bla activity in the absence and presence of the inhibitors. The efficiency of the enzyme activity inhibition can be screened based on the specific colour changes.

After obtaining the precursor, the β -lactam substrate was used to determine the enzymatic activity *in vitro*. In a typical experiment, substrate (8.0 μ M) was initially incubated with transformed TEM-1 Bla (2.0 nM) in a PBS buffer (pH 7.4) in the absence of an inhibitor for 20 min. The resulting solution was subsequently transferred into Au-NPs suspension (15 nm, 2.5 nM). As shown in Fig. 1(a), a significant colour change



Scheme 1 Bla inhibition assay based on color changes in the presence and absence of Bla inhibitors during enzyme-induced Au-NPs aggregation.

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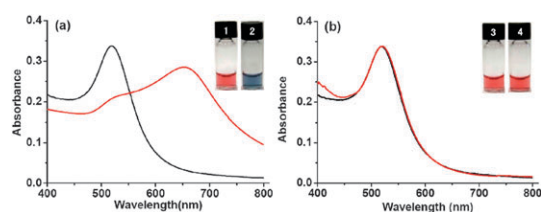


Fig. 1 Colorimetric assay for Bla inhibition. (a). UV-vis spectra of Au-NPs before (black) and after (red) incubation with Bla-treated substrate in the absence of an inhibitor. (b). Similar test as (a) but in the presence of an inhibitor (2.0 μM). The inset shows the colour change of Au-NPs. 1: Au-NPs only; 2: Au-NPs, Bla, and substrate; 3: Au-NPs only; 4: Au-NPs, Bla, inhibitor, and substrate.

from red to blue occurred within seconds. Both a decreased absorbance at 520 nm and an increased absorbance at 650 nm were observed in the UV-vis spectrum as time increased. Control experiments under different pH and various concentration of PBS buffer (see ESI, Fig. S1[†]) demonstrated that the colour change and spectrum shift of Au-NPs suspension was mainly due to the monomeric β -lactam substrate and Bla interaction. On the basis of the colour change and spectrum shift, Bla concentration down to 1.0 pm can be easily quantifiable with substrate and Au-NPs (see ESI, Fig. S2[†]), which is more sensitive than the colorimetric sensor as reported recently.⁹ As expected, in the presence of the efficient Bla inhibitor (e.g., tazobactam), no detectable colour change and spectrum shift occurred after the addition of Bla-treated substrate (8.0 μM) into Au-NPs (Fig. 1(b)). The Bla activity was decreased significantly and the suspensions were stable without showing signs of aggregation. The IC₅₀ values of the enzyme inhibitors were estimated accordingly and the results were in agreement with the inhibitor activities as determined by using a standard indicator: nitrocefin (see ESI, Fig. S4 and S5[†]).

The different aggregation induced by the enzymatic reaction in the absence and presence of an efficient inhibitor was also monitored by transmission electron microscope (TEM). As shown in Fig. 2(a)–(c), the enzyme interaction induced the conjugation of amino-thiol modified linker to the surface of the Au-NPs and resulted in the dramatic aggregation. In the presence of a sufficient amount of inhibitor (2.0 μM), no

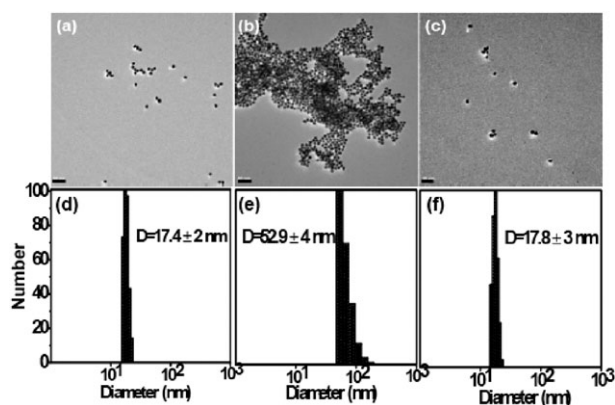


Fig. 2 TEM images (above) and hydrodynamic diameter distribution plots as determined by DLS measurements (below) for (a), (d) substrate (8.0 μM) in Au-NPs only; (b), (e) incubation of substrate (8.0 μM) with Bla in the absence and (c), (f) presence of inhibitor tazobactam (2.0 μM) in Au-NPs solutions. Scale bar: 50 nm.

significant binding of Au-NPs to each other could be observed, which is similar to the results in the Au-NPs suspension without enzyme treatment. Dynamic light scattering (DLS) measurements was also performed to determine the sizes and size population distributions of Au-NPs. The data shown in Fig. 2(d)–(f) further indicated that the interaction between substrate and enzyme was crucial for the aggregation of Au-NPs, which is consistent with the results in TEM assays.

In order to indicate the potential of this method for the parallel screening of different inhibitors, all reactions were transferred to a multi-well microplate, and the efficiency of enzyme inhibition, as a proof of concept, was evaluated with the addition of different Bla inhibitors: aztreonam (ATM), clavulanate acid (CA), tazobactam (TZB), and sulbactam (SUL). Most of them have been recognized as inhibitors to efficiently suppress class A Bla activities in clinics. As shown in Fig. 3, visualization of different solution colours or detection of absorbance ratio ($A_{650\text{ nm}}/A_{520\text{ nm}}$) in the microplate generates valuable information on the efficiencies of the four inhibitors for β -lactamase inactivation. A red solution reveals the potent enzyme inhibition, while a blue solution indicates the least inhibition and aggregation of Au-NPs induced by the enzymatic reaction. The inhibitors decreased the Bla activities and exhibited the following trend in enzyme inhibition: TZB > CA > SUL > ATM. This is consistent with the result by using nitrocefin when the inhibitor concentrations are larger than 3.0 μM (see ESI, Fig. S3[†]). This order is also similar to the reported binding affinities of different inhibitors to the different types of class A β -lactamases.^{4a} No colour and absorbance ratio ($A_{650\text{ nm}}/A_{520\text{ nm}}$) difference among the inhibitor activities can be observed at low inhibitor concentrations in the nitrocefin assay (Fig. 3(a)). The results suggest that Au-NPs-based inhibition assay may lead to a more efficient manner to effectively screen β -lactamase inhibitors *in vitro*.

To evaluate the efficiencies of the different inhibitors in the bacteria, four β -lactam resistant bacterial strains: TEM-1 transformed *E. coli* Bl21, TEM-1 *E. coli* (ATCC 35218), *Bacillus cereus* (ATCC 13061), and *K. pneumoniae* (ATCC 700603),¹⁰ which contained different kinds of class A Bla such as transformed

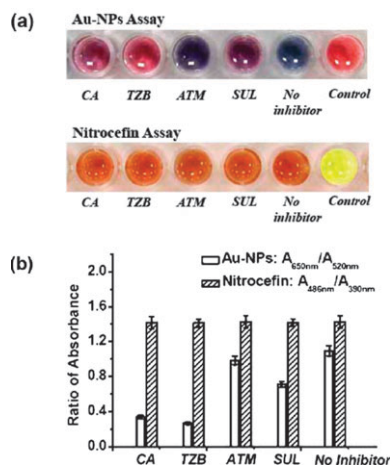


Fig. 3 (a) Visualization of *in vitro* Bla inhibition in Au-NPs (2.5 nM) and nitrocefin (20 μM) assays at a TEM-1 Bla concentration of 2.0 nM. (b) Absorbance change ratio of Bla inhibition assay in the absence and presence of different inhibitors (inhibitor concentration: 0.1 μM).

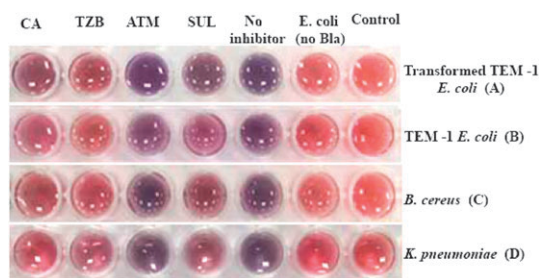


Fig. 4 Au-NPs (2.5 nM)-based colorimetric assays for Bla inhibition in a 96-well microplate with different combinations of four inhibitors and four class A Bla contained living bacteria (A: transformed TEM-1 *E. coli* BL21, $\sim 10^8$ cfu mL $^{-1}$, B: TEM-1 *E. coli*, $\sim 10^9$ cfu mL $^{-1}$, C: *Bacillus cereus*, $\sim 8 \times 10^9$ cfu mL $^{-1}$, and D: *K. pneumoniae*, $\sim 3 \times 10^8$ cfu mL $^{-1}$). Bacteria without inhibitor as positive control. Wild type *E. coli* BL21 (no Bla) and Au-NPs solutions as negative controls (inhibitors concentration: 0.1 μ M).

TEM-1, TEM-1, PenPC, and SHV-18, respectively, were chosen and the enzyme activities were identified in the absence and presence of Bla inhibitors. Wild type *E. coli* BL21, which could not express Bla, was used as a negative control. As shown in Fig. 4, the clear colour change in plasmid-transformed *E. coli* BL21 exhibited the trends similar to those observed in *in vitro* measurement. CA and TZB are comparable in this bacterial strain. A red colour in TZB indicated the relatively potent Bla inhibition, a reddish colour in CA exhibited a weaker inhibition activity than that in TZB but stronger than that in SUL. A blue colour in ATM, which was close to the solution without inhibitor treatment, demonstrated the least activity for the enzyme inhibition. The absorbance ratios at 650 nm and 520 nm also confirmed the same inhibition order (TZB > CA > SUL > ATM) as observed in colour change (see ESI, Fig. S7).[†] Similar results were obtained in TEM-1 *E. coli*, and *Bacillus cereus* strains, although a large amount of bacterial strains had to be used due to the lower enzyme activities in these two bacteria. We also tested the enzyme inhibition screening by using *K. pneumoniae*, one clinically isolated β -lactam resistant bacterial strain, which contained the extended-spectrum β -lactamase (ESBL): SHV-18. A red colour in CA demonstrated the efficient enzyme inhibition, which was more effective compared to TZB. This is in accordance with the known activities of these inhibitors in *K. pneumoniae*.¹⁰ Similarly, the blue in ATM suggested weak enzyme inactivation. This result demonstrated that different inhibitors would exhibit various activities toward the same bacterial enzyme. Furthermore, the different Bla inhibitions observed in *E. coli*, *Bacillus cereus*, and *K. pneumoniae* strains were attributed to the various inhibition activities of the same inhibitor to the different enzymes in bacteria. As control, the enzyme inhibitor screening was also conducted by using nitrocefin assay (see ESI, Fig. S6).[†] No difference in colour change was observed when the inhibitor concentration was 0.1 μ M. The significant colour change with respect to the enzyme inhibition could only be identified after the addition of a large amount of inhibitors (>3.0 μ M). The trends in colour change were identical to the results as determined by using Au-NPs. The lower inhibitor concentrations used in Au-NPs-based colorimetric method indicated a higher reporting threshold, which could lead to an alternative

approach to screen class A β -lactamase inhibitors in a more efficient manner compared to nitrocefin assay.

In summary, we have developed a simple and effective Au-NPs-based colorimetric method for efficient screening of class A β -lactamase activity and inhibitors both *in vitro* and in bacterial strains without requirement of sophisticated instruments. The colorimetric method based on the aggregation of Au-NPs can be used not only to sensitively identify the enzyme activity, but also to provide valuable information on the efficiencies of simultaneous screening of different enzyme inhibitors *in vitro* and in the varied enzyme expressed bacteria. This simple screening method may offer an alternative platform to study the inactivation of β -lactam antibiotics for the approach that counteract antibacterial drug resistance; it may also open the doors for the future application of nanoparticles-based technologies in the field of drug-development.

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