

A Simple and Specific Assay for Real-Time Colorimetric Visualization of β -Lactamase Activity by Using Gold Nanoparticles**

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β -Lactamases (Blas) are a family of bacterial enzymes that can hydrolyze the β -lactam ring in penicillins and cephalosporins with high catalytic efficiency and render the bacteria resistant to the β -lactam antimicrobial reagents.^[1] Currently, increased resistance of bacterial infections to antibiotic treatment has been extensively documented and has become a generally recognized problem for clinicians worldwide, in both hospital and community settings.^[2] Therefore, detecting Blas and screening their inhibitors in biological samples before conducting the efficient antibiotic therapy is extremely important clinically. Some commonly used fluorescent (for example, genotyping based on the polymerase chain reaction (PCR)) or chromogenic assays (such as nitrocefin or pyridine-2-azo-*p*-dimethylaniline cephalosporin (PADAC) indicators) are currently used successfully to perform such detections.^[3] Some other fluorogenic substrates and biocompatible hydrogels have also been developed as reporters for imaging the gene expression of Blas in vitro and in vivo.^[4] A simple, rapid, and economical assay is still highly desirable because current assays have some major drawbacks, such as laborious manipulation, time-consuming processes, high reliance on specific instrumentation, and limited chemical stability and aqueous solubility of the individual substrates.

Gold nanoparticles (Au-NPs) have interesting optical and electronic properties that have served as a versatile platform for exploring many facets of basic science. Au-NPs can exhibit plasmon coupling that may red-shift the resonance wavelength.^[5] Normally, the exact plasmon resonance band can be determined by the size, shape, medium, and the relative distance between the particles.^[6] The different agglomeration states of Au-NPs can result in distinctive color changes. This extraordinary optical phenomenon makes Au-NPs ideal chromogenic probes for reporting molecular recognition events,^[7] such as making a molecular ruler for colorimetric detection of DNA hybridization,^[8] carbohydrate sensing,^[9]

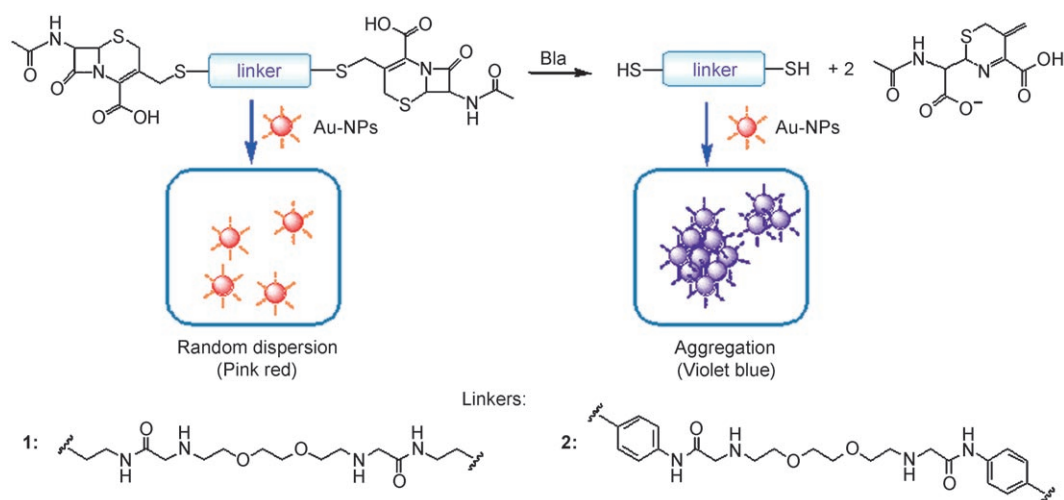
sensitive measurement of metal ions,^[10] optical study of a single virus,^[11] and monitoring special enzyme activities such as alkaline phosphatases, kinases, proteases, etc.^[12] Currently, most of Au-NP-based colorimetric assays for enzyme detection are mainly dependent on the enzyme properties to induce the change in gold-particle aggregates. One design to achieve this target is to monitor the Au-NP agglomeration states from aggregation to dispersion. In this case, enzymes react with DNA or peptide-functionalized nanoparticles and induce their disassembly. The dispersion of Au-NP aggregates leads to significant color changes. This type of colorimetric assay has been proved to be simpler, more direct and accurate, and has been successfully used to sensitively determine the presence of the enzymes in real time.^[8d,12d] One aspect that needs to be improved for this assay is the steric hindrance and slow enzyme kinetics caused by the bulky conjugation of enzyme substrates with Au-NPs. An alternative design for the Au-NP-based colorimetric assay relies on the assembly process of dispersed gold particles. In this detection system, enzymes react with free substrates first. The released products are then able to cross-link the Au-NPs, which leads to aggregates. This type of assay is less sterically hindered, exhibits fast enzyme kinetics, and is more efficient in monitoring the enzyme activity without functionalization of the Au-NPs.^[12a,b] We have developed a novel and easily operational chromogenic assay based on Au-NP aggregates to detect Bla activity in vitro and in antibiotic-resistant bacterial suspensions. This method can be used to rapidly identify the Bla molecules and screen the enzyme inhibitors in a high-throughput fashion by the naked eye or a simple colorimetric reader.

Scheme 1 illustrates the simple procedure of using Au-NPs to detect Bla. Cleavage of the β -lactam ring in a cephalosporin triggers spontaneous elimination of any leaving groups previously attached to the 3'-position. Here, two cephem nuclei are connected through a dithiol-modified 1,2-bis(2-aminoethoxy)ethane flexible linker after iodo substitution. The thiol group is an excellent leaving group and will facilitate fragmentation upon enzyme treatment. Another advantage of the introduction of a thiol group is that its strong interactions with gold surfaces lead to the aggregation of gold nanoparticles. 1,2-Bis(2-aminoethoxy)ethane was used here to improve the substrate solubility and to minimize the steric interactions between the substrates and the enzyme. To optimize the kinetic properties of the substrates, two different thiol groups, 2-mercaptoethylamine- and 4-aminothiophenol-conjugated 1,2-bis(2-aminoethoxy)ethane linkers, were connected to the 3'-position of the cephem nucleus. A simple deprotection (that is, removal of the diphenyl methyl ester at the 4'-position by trifluoroacetic acid and anisole) followed by

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[**] This work was supported by SEP (RG139/06) and URC (RG56/06) grants at the Nanyang Technological University. We thank Prof. P. K. Wong and Dr. Z. Y. Zhong at ICES for the TEM measurement and Dr. Edwin K. L. Yeow, Dr. Brendan P. Orner, and the reviewers for helpful suggestions.

Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.



Scheme 1. Design of the Bla assay based on color changes during enzyme-induced Au-NP aggregation.

HPLC purification produced the substrates **1** and **2** with yields of 60 and 66.7 %, respectively.

After obtaining the precursors, we tested the ability of Bla activity to induce the aggregation of Au-NPs. Substrates (8 μ M) were initially incubated with Bla (5 nM) in phosphate-buffered saline (PBS) buffer solution (pH 7.4) for 20 minutes. Then, the resulting solutions were transferred into Au-NP suspensions (15 nm in size and with a concentration of 2.6 nM as determined by the reported method).^[13] To stabilize the nanoparticles and also to prevent the interaction of proteins with gold particles, 0.1 % poly(ethylene glycol) (PEG) 8000 was added to the reaction mixture.^[12a] As shown in Figure 1 a,

the color of the Au-NP suspension alone remained unchanged with time. When the intact substrates were added, no further color changes in the Au-NPs demonstrated that both of the β -lactam substrates were stable under the experimental conditions. In the presence of the Bla-pretreated substrate **1**, no color change was detected within 30 minutes. A clear change was only observed after a longer incubation time (> 9 h; data not shown). In contrast, after adding the Bla-pretreated substrate **2** into the Au-NPs, the color dramatically changed from pink red to violet blue within seconds. Significant changes in the UV/Vis absorption were also identified 30 minutes after mixing the enzyme-treated substrate **2** with the Au-NPs (Figure 1 b). Both a decreased absorbance of the plasmon band at 520 nm and an increased absorbance at 650 nm were observed with increasing time. The shifting of the absorbance to a longer wavelength over time correlated with the color change seen in Figure 1 a.

The Bla-induced Au-NP aggregates were used to monitor the process of the enzymatic hydrolysis reactions. Figure 2 a shows the time course for the color change of the gold particles upon the addition of Bla-pretreated substrates. For substrate **1**, the absence of a significant color change within 30 minutes demonstrates the slow process of the overall enzyme assay. For substrate **2**, about two thirds of the change in the total absorbance occurred within the first five minutes, thereby confirming that this reaction process was very fast. We hypothesized that the colorimetric assay mainly consisted of a two-step interaction: one was the enzymatic hydrolysis to release the dithiol linkers and the other was the binding of cleaved dithiol linkers to the surface of Au-NPs to form an aggregation. Figure 2 b shows the change in the absorbance recorded for the binding of free dithiol linkers to the Au-NPs at different times (linker **1**: di-2-mercaptoethylamine-conjugated 1,2-bis(2-aminoethoxy)ethane and linker **2**: di-4-aminothiophenol-conjugated 1,2-bis(2-aminoethoxy)ethane). Both of the linkers exhibited similar affinities to that of the Au-NPs, and their rapid binding process, which occurred within seconds, suggested that the kinetics of the system was limited to an enzymatic hydrolysis reaction. Therefore, the colorimetric feature of Au-NPs was applied to measure the

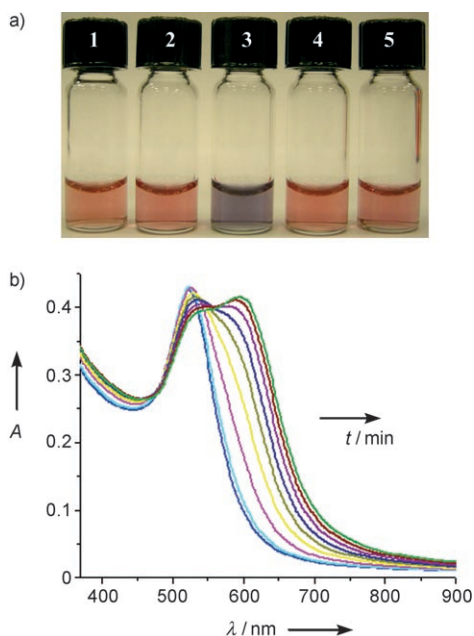


Figure 1. Colorimetric assay of Bla. a) Colors of the Au-NP solution in the absence or presence of Bla-treated substrates; 1: Au-NPs only; 2: Au-NPs and substrate **2**; 3: Au-NPs and Bla-treated substrate **2**; 4: Au-NPs and substrate **1**; 5: Au-NPs and Bla-treated substrate **1**. b) UV/Vis spectra of Au-NPs taken at 2-min intervals for 30 min after the addition of Bla-treated (5 nM) substrate **2** (8 μ M).

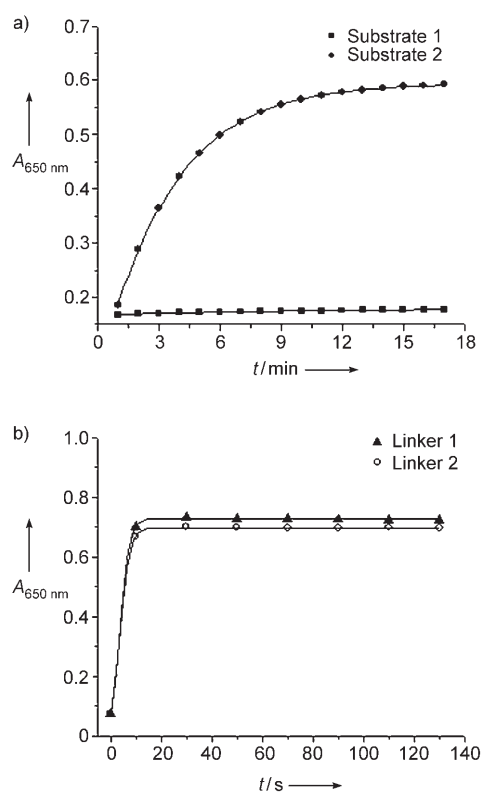


Figure 2. Aggregation kinetics of Au-NPs for Bla. a) Absorbance change (at 650 nm) of Au-NP suspension with time in the presence of Bla-pretreated substrates (5 μM): substrate 1 (■), substrate 2 (●). b) Dependence of absorbance change on time for the interactions of free dithiol linkers (5 μM) with the Au-NP suspension: linker 1 (▲), linker 2 (○).

hydrolysis kinetics of Blas. Further analysis of the enzymatic kinetics of the two substrates at pH 7.4 allowed the determination of $k_{\text{cat}} = (0.33 \pm 0.1) \text{ s}^{-1}$ and $K_{\text{M}} = (140 \pm 11) \mu\text{M}$ for substrate 1 and $k_{\text{cat}} = (8.69 \pm 1.3) \text{ s}^{-1}$, $K_{\text{M}} = (113 \pm 8.0) \mu\text{M}$ for substrate 2 (see the Supporting Information). The small value of k_{cat} for substrate 1 demonstrated that the leaving group based on the alkyl thiol group was less efficient than that of thiolphenol which was used in substrate 2. This result suggests that the alkyl thiol based substrate 1 was itself kinetically inert towards Bla and thus exhibited a slow response to Au-NP aggregation. A concentration as low as 60 pM of Bla is readily detectable with substrate 2 and Au-NPs (see the Supporting Information), which is more sensitive than that of the commonly used Bla indicator, nitrocefin.^[14] This detection sensitivity can be profoundly influenced by the concentrations of Au-NPs. Figure 3 shows the quantitative relationship between the absorbance change ($(A - A_0)/A_0$) at 650 nm and the different concentrations of gold particles towards 60 pM Bla. It is clear that the significant change in the absorbance at the Au-NP concentrations ranging from 1.0 to 4.0 nM results in a good sensitivity for the colorimetric enzyme assay for Bla. The largest change in absorbance from dispersed to fully aggregated nanoparticles was observed when the concentration of Au-NPs was approximately 2.6 nM.

We also determined the Bla inhibition by using this simple and sensitive Au-NP assay. To demonstrate this application,

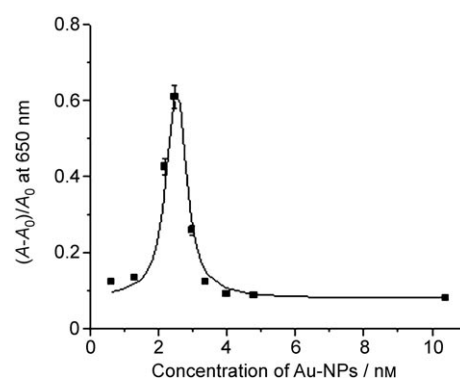


Figure 3. Absorbance change at 650 nm of Au-NPs at 2 h after mixing 60 pM Bla-pretreated substrate 2 (8 μM) with various concentrations of gold particles (ranging from 0.65, 1.3, 2.2, 2.6, 3.0, 3.4, 4.0, 4.8, to 10.4 nM; analyses were performed in triplicate).

one commonly used Bla inhibitor, sulbactam, was selected and the effect of the enzyme inhibition was evaluated by using our system. After incubating the inhibitor-pretreated Bla with substrate 2 and then mixing with Au-NPs, a different color was observed, which indicated the different properties of the enzyme inhibition. The IC_{50} value was found to be about 4.4 μM , which was similar to the previously observed value (Figure 4).^[15] This finding supports our hypothesis that the color changes can be used for a quantitative analysis of Bla activity and for screening the Bla inhibitors.

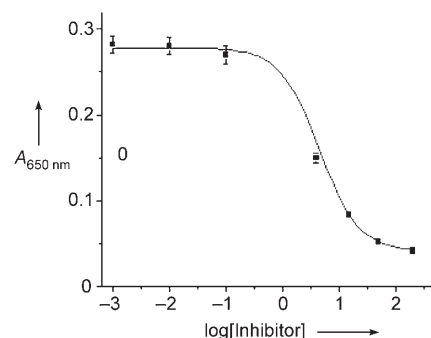


Figure 4. Inhibition assay of Bla activity by sulbactam. The reaction was monitored by the absorbance change at 650 nm (analyses were performed in triplicate).

Furthermore, the different aggregation behaviors of substrate 2 with Bla were monitored by TEM. As shown in Figure 5a, substrate 2 itself (8 μM) was unable to induce the aggregation of the Au-NP suspensions. Upon treatment of the same concentration of substrate 2 with Bla (5 nM), the enzyme interaction triggered the release of the modified dithiol linker, thus inducing the cross-linking of the Au-NPs and increasing the aggregation dramatically (Figure 5b). Although some agglomerations could also be detected in Figure 5a, possibly caused by self-assembly during the drying process in the sample preparation, most of the Au-NPs were dispersed randomly in the solution with diameters of approximately 15 nm. Dynamic light scattering (DLS) measurements further

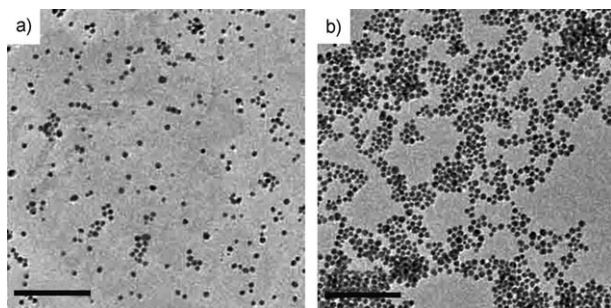


Figure 5. TEMs of a) substrate **2** (8 μm) in Au-NPs only and b) incubation of substrate **2** (8 μm) with Bla (5 nm) in Au-NP solutions. Scale bars: 200 nm.

confirmed a well-dispersed population of Au-NPs in the solution with substrate **2** only and highly aggregated Au-NPs in the solution with Bla-pretreated substrate **2** (see the Supporting Information).

To evaluate whether substrate **2** would respond to Bla in living bacterial cells, different strains of bacteria (ca. 10^8 cfu mL $^{-1}$; cfu = colony-forming units) such as wild-type *E. coli* Bl21, antibiotic-resistant plasmid-encoded *E. coli* Bl21, and one clinically isolated β -lactam-resistant *K. pneumoniae* (ATCC 700603) were incubated with substrate **2**. As shown in Figure 6a, substrate **2** treated with wild-type *E. coli* Bl21 do

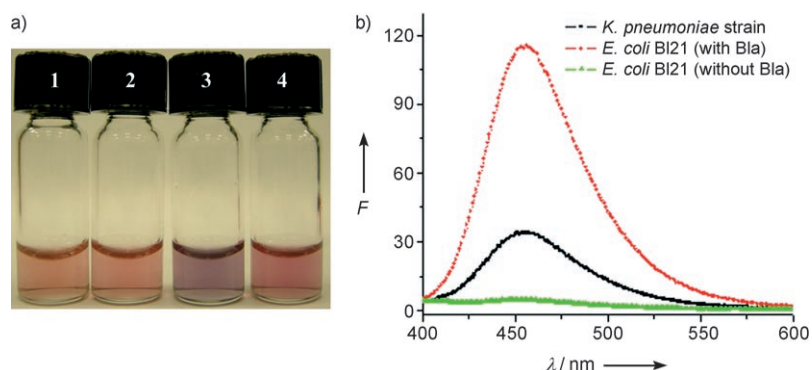


Figure 6. a) Colorimetric assay of the Au-NP aggregation at 30 min after incubation with bacterial strains; 1: Au-NPs only; 2: substrate **2** (8 μm) treated with wild-type Bl21. 3: substrate **2** treated with plasmid-encoded Bl21; 4: substrate **2** treated with *K. pneumoniae*. b) Fluorescent emission (I_f) of CC1 (2 μm) in wild-type *E. coli* Bl21 (green), *K. pneumoniae* (black), and plasmid-encoded *E. coli* Bl21 (red). The excitation was measured at 360 nm.

not lead to the Au-NPs color change because it is unable to express Bla. However, significant color change was observed within 30 minutes after adding plasmid-encoded *E. coli* Bl21 or substrate **2** treated with *K. pneumoniae* to the Au-NP suspensions. The different color changes (violet blue in Bla-encoded *E. coli* Bl21 and reddish purple in *K. pneumoniae*) were attributed to the different Bla expressions. These two β -lactam-resistant bacterial cells contained different kinds of extended-spectrum β -lactamases (ESBLs; TEM-1 in plasmid-encoded *E. coli* Bl21 and SHV-18 in *K. pneumoniae*).^[16] The

different types of Blas exhibited different enzymatic conversions for the same substrate, which is consistent with results reported recently.^[4d] A CC1 fluorescent assay was performed to determine Bla activity in these bacterial strains (see the Supporting Information).^[4b] As shown in Figure 6b, no significant fluorescent signal was detected in wild-type *E. coli* Bl21. Emission of CC1 in *E. coli* Bl21 (with Bla) was approximately four times higher than that in *K. pneumoniae*, which confirmed the highest enzyme activity in the Bla-(TEM-1)-encoded *E. coli* Bl21 strains. As a commonly used fluorogenic probe, CC1 was more sensitive in the detection of Blas than was the colorimetric assay. However, CC1 itself was not as stable and spontaneous hydrolysis easily occurred. In addition, the whole fluorescent assay had to be conducted with specific instrumentation, such as with a fluorometer or a fluorescent microscope.

The colorimetric assay was also performed using nitrocefin, a standard Bla indicator (see the Supporting Information). The result indicated that nitrocefin could induce a similar color change from yellow to pink in both of the β -lactam-resistant bacteria. There was no color difference between the Bla-encoded *E. coli* Bl21 and the *K. pneumoniae* strains. Moreover, the pink color was also detected in the wild-type *E. coli* Bl21 bacteria where no Bla was present, which is possibly because of the nonspecific hydrolysis of nitrocefin. Therefore, compared with the nitrocefin-based colorimetric assay, the significantly different color change observed in different β -lactam-resistant bacteria and the absence of background activity in wild-type bacterial strains in the Au-NP-based colorimetric assay indicate that the latter has a higher reporting threshold than that of the nitrocefin assay. This finding suggests that the Au-NP-based enzymatic assay provides a particularly useful reporting approach for systems that have significant background activity, which would cause a false-positive signal in the nitrocefin assay. This new Au-NP-based colorimetric assay induced by an enzymatic reaction leads to an alternative approach for the simple and specific indication of living drug-resistant bacteria in real time.

In summary, the work presented herein describes a simple, economical assay to rapidly visualize Bla activities by using gold nanoparticles. This assay, which involves the use of Bla to cleave the modified dithiol linker from the cephem nucleus and to induce the cross-linking of Au-NPs, does not require specific instrumentation or complicated experimental steps. It can offer an alternative platform to evaluate the enzymatic kinetic reactions and to screen Bla inhibitors in real time. It may also provide useful practical applications for the rapid and specific detection of antibiotic-resistant bacteria in clinical settings. Development of a new type of the Au-NP-based colorimetric β -lactam substrates with small molecular weights, efficient cell permeability, and fast pharmacokinetic properties is currently ongoing.

Experimental Section

Detailed experimental procedures for the syntheses of the substrates as well as their characterization are given in the Supporting Information.

Colorimetric assay for Bla: Substrate solutions were prepared in deionized water and Bla was dissolved in PBS buffer solution (pH 7.4) to make different Bla concentrations. Then, Bla solution (10 μ L) was mixed with the substrates (190 μ L) for the enzyme interactions. The Bla-pretreated substrates were transferred into a Au-NP suspension (800 μ L) that contained 0.1 % PEG 8000. The final substrate concentrations were maintained at 8 μ M. The enzymatic reaction was performed by incubating the different Bla concentration with substrates for 20 min at room temperature. All the tests were performed in triplicate.

Colorimetric visualization of living bacterial cells: Bacterial cells (ca. 10^8 cfu mL⁻¹) were suspended in deionized water (200 μ L) that contained the substrates at room temperature. The suspension was incubated for 20 minutes to enable further enzyme interactions. After centrifugation, the supernatant was tested for a colorimetric image. The bacterial solution (200 μ L) was added into an Au-NP suspension (containing 0.1 % PEG 8000; 800 μ L) to induce the aggregation of Au-NPs. The color change of the Au-NPs was recorded at different time intervals.

Received: June 23, 2007

Revised: August 22, 2007

Published online: October 17, 2007

Keywords: β -lactamase · bacterial cells · colorimetric assays · enzymes · nanoparticles

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