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A simple design of fluorescent probe for indirect detection of β-lactamase based on AIE and ESIPT processes

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A novel fluorescent probe **DNBS-CSA** is developed for light-up detection of β-lactamase. The probe design is based on an indirect detection approach with three step reactions. β-Lactamase can react with the lactam of its substrate (cefazolin sodium) to produce a secondary amine, initiating a spontaneous elimination reaction and affording a thiol compound. The thiol could further react with the sulfonate group of **DNBS-CSA**, releasing the salicylaldehyde azine derivative (**CSA**) with both aggregation induced emission (AIE) and excited-state intramolecular proton transfer (ESIPT) characteristics. Previously reported β-lactamase probes require covalent linkage of the substrate β-lactam ring part to probe, which makes the probe synthesis difficult due to the complicated structure of β-lactam ring. On the contrary, modification of β-lactam ring is no longer necessary for **DNBS-CSA** according to our indirect detection approach. The linear range of fluorescent quantification for β-lactamase is 0-10 mU/mL in aqueous solution. Moreover, owing to the AIE property of **CSA**, detection of β-lactamase with **DNBS-CSA** on test papers was also achieved.

Introduction

β-lactamase is an important bacterial enzyme that can cleave the amide group of β-lactam antibiotics[1-3] (penicillins, cephalosporins, etc.) with high catalytic efficiency, which makes the β-lactamase producing bacteria, such as Gram-negative bacterium[4] and Staphylococcus[5], resistant to β-lactam antibiotics. Thus, β-lactamase is a significant biomarker for identification of bacterial pathogens that are resistant to β-lactam antibiotics.[6] In addition, there are reports[7, 8] on illegal addition of β-lactamase into milk to conceal the antibiotics residue by unethical manufacturers, in order to reuse the contaminated milk illegally. Therefore, the sensitive detection of β-lactamase is of great importance.

Fluorescent probes for various analytes have attracted much attention owing to the simplicity, low cost, good sensitivity, and capability of real-time detection, which enable effective in-vitro and in-vivo studies.[9-22] So far, many fluorescent probes for enzyme activity detection have been reported, such as esterase[23, 24] and alkaline phosphatase probes[25] based on hydrolisis of ester group, pantetheinase probe based on cleavage of pantetheine,[26] nitroreductase probe based on reduction of nitro group.[27] However, only limited probes for β-lactamase have been developed.[3, 28-31] The scarcity of β-lactamase probe is mainly due to the difficulty of probe synthesis. Compared with the substrates of other enzymes, the substrate (β-lactam ring) of β-lactamase has a much more complicated structure. The previously reported β-lactamase probe all adopted a direct detection approach, requiring tedious modification of substrate (β-lactam ring) to probe. Thus, it is still very challenging to develop novel detection approach for β-lactamase, which should not only match up with the requirement of sensitivity, detection time and ease of real sample analysis, but also avoiding the complex modification of β-lactam ring.

Moreover, the reported fluorescent probes for β-lactamase are designed with fluorophores suffering from aggregation caused quenching (ACQ),[32, 33] such as indolium and fluorescein, which limited their application when used in low β-lactamase content detection since fluorescence signal could not be elevated by increasing probe concentration. Besides, due to the ACQ effect, it is hard to apply these probes for β-lactamase detection in solid state, such as on test papers, despite that paper-based sensors[34, 35] could find potential application in point-of-care detection due to their portable, inexpensive, disposable and low sample volume requirement. Therefore, developing β-lactamase probes that allows detection in solid state without ACQ effect is still needed.

Fluorophores with aggregation induced emission (AIE)[36-43] are a series of molecules emitting strong fluorescence in their aggregate or solid states. Up to now, many AIE fluorophores based on hydrocarbons, heteroatoms or organometallics have been constructed as fluorescent materials for biosensing applications.[40, 44-46] Previously, we have developed a class of AIE fluorophores based on salicylaldehyde azines (SA)[47] with excited state intramolecular proton transfer (ESIPT)[48, 49] mechanism. The hydroxyl groups in these molecules are

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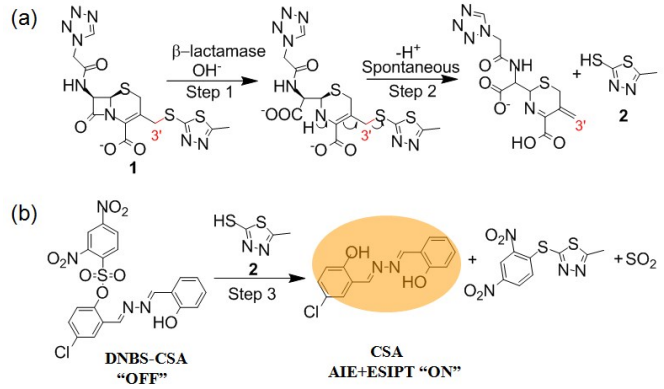
responsible for ESIPT and essential for their AIE characteristics in aggregate and solid states. Therefore, a universal SA-based AIE probe design strategy were proposed through protection and deprotection of the hydroxyl groups. Based on this strategy, we have developed several fluorescent probes for the detection of thiol,[50] beta-galactosidase[51] and esterase,[52] demonstrating the versatility of the platform.

Herein, we report a simple design of fluorescent probe for indirect detection of β-lactamase based on AIE and ESIPT processes. The indirect detection approach incorporates three-step reactions (Scheme 1): firstly, a cephalosporin substrate **1** of β-lactamase was introduced into the detection system to cleave the β-lactam ring and release a secondary amino group; secondly, the amino group further triggers spontaneous elimination reaction to afford an intermediate product **2** containing thiol; and thirdly, the thiol of **2** could further react with the sulfonate group of probe **DNBS-CSA** to release the essential hydroxyl group, recovering the AIE fluorescence. Thus, the activity of β-lactamase could be detected indirectly by our previously reported thiol probe **DNBS-CSA**[50]. This indirect detection approach does not need tedious modification of β-lactam to probe, while strong yellow AIE fluorescence was observed in the presence of β-lactamase, allowing the light-up detection of β-lactamase activity both in solution and on test papers.



Scheme 1 An indirect approach for fluorescent light-up detection of β-lactamase by using probe **DNBS-CSA**.

Experimental

Materials and instruments

β-Lactamase from Enterobacter cloacae and other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) and used without further purification. Plain milk was used as real sample without further pretreatment.

All fluorescence spectra were determined on a fluorescence spectrometer (JASCO FP 8600, Japan). All NMR spectra were recorded using a NMR spectrometer (JEOL JNM-ECA300, Japan). The mass spectra were obtained on an ion trap time-of-fight mass spectrometry (Shimadzu MS-IT-TOF, Japan). The pH measurement was performed with a pH meter(Sartorius PB-10 basic, Germany).

Synthesis of compound DNBS-CSA and CSA

Compound **DNBS-CSA** and **CSA** were prepared according to our previously reported procedure.[50] The molecular structures were confirmed to be right by the NMR and MS spectroscopy (details can be found in Supplementary Information).

Analytical procedures

The stock solution (500 μM) of **DNBS-CSA** was prepared in DMSO. Stock solutions of cefazolin sodium salt (8.0 mM), β-lactamase (10.0 U/mL) and other analytes ($CaCl_2$, $Mg(NO_3)_2$, $Zn(NO_3)_2$, $Fe(NO_3)_3$, $Cu(NO_3)_2$, pepsin, lysozyme, vitamin C, alanine, BSA, lactose, casein, and esterase) were prepared in PBS buffer (10 mM, pH 7.4), respectively. In a typical detection procedure, 150 μL **DNBS-CSA** stock solution and 300 μL cefazolin stock solution were added into a test tube. Subsequently, a proper amount of β-lactamase stock solution was added, making the total volume of sample solution to be 500 μL. After reaction at 37 ℃ for different time, the fluorescence spectra were recorded.

For the β-lactamase sensing in real samples, test paper and milk sample solution were prepared, respectively. By dropping 5 μL solution of **DNBS-CSA** in THF (10 mM) on the filter paper strip and underwent evaporation of THF for 3 min, test paper was fabricated. In a typical procedure, sample solution containing 10% milk was prepared by adding 600 μL cefazolin stock solution (8.0 mM), 100 μL milk, different volume of PBS buffer and β-lactamase stock solution (10.0 U/mL or 0.1 U/mL) into a test tube, making the total volume of sample solution as 1000 μL. The test paper was subsequently dipped into the sample solution. After incubation at 80 ℃ for 20 min, the test paper was taken out and dried for 30 min. Photographs of test paper was taken under a UV lamp and the Image J software was used to read the intensity of fluorescent spot on test paper. The β-lactamase concentration in sample solution was then calculated through the calibration curve.

Results and discussion

Probe design

As shown in Scheme 1, **DNBS-CSA** was synthesized for indirect detection of β-lactamase. In the structure of **DNBS-CSA**, one hydroxyl group was blocked by sulfonate and thus the ESIPT process was restricted. Besides, the nitro groups on benzene sulfonate could serve as effective quencher. Therefore, **DNBS-CSA** was initially non-fluorescent. After the addition of β-lactamase, it cleaved the β-lactam ring of the cefazolin substrate and created a free secondary amino group, which triggered spontaneous elimination of the leaving group previously attached to the 3' position[1] (Scheme 1a). Thus, an intermediate product **2** containing thiol was afforded, which further cleaved the sulfonate group of **DNBS-CSA,** removing the 2, 4-dinitrobenzenesulfonate group and recovering the hydroxyl group, yielding **CSA**. While in **CSA**, two essential hydroxyl groups were present that enabled the ESIPT and the AIE fluorescence. In this way, an indirect fluorescent light-up detection method for β-lactamase was established.

The AIE characteristic of CSA

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We firstly studied the effect of water volume fraction (f_w= 0-99 vol%) on the fluorescence of **CSA** in water/DMSO co-solvents, and the results are shown in Fig. 1. **CSA** (150 μM) is soluble in DMSO but is not in water. In a good solvent DMSO, **CSA** disperses well with no fluorescence. However, in a poor solvent (f_w= 99 vol%), an intense fluorescence band at 558 nm can be observed. The AIE effect of **CSA** occurs when the water volume fraction in the co-solvent is more than 30% and fluorescence intensity could be further enhanced with higher water volume fractions, which correlates well with the increased aggregates formation in poorer solvents. The AIE fluorescence is originated from restriction of free intramolecular rotation assisted by intramolecular hydrogen bonds of **CSA** in the aggregate state and from the excited state intramolecular proton transfer (ESIPT)[48-50].

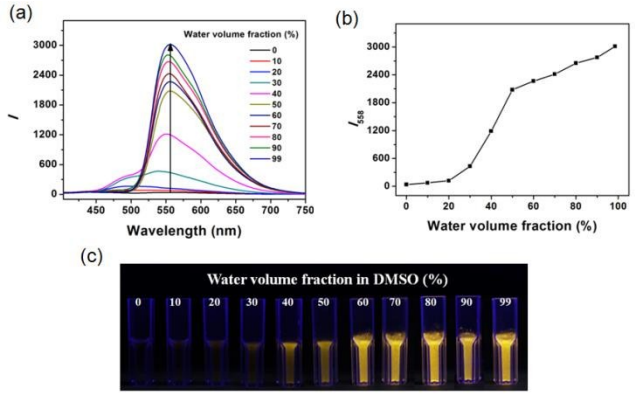

Fig. 1 (a) Effect of water volume fraction on the (a) fluorescence spectra and (b) intensity (I_{558}) of **CSA** (150 μM) in water/DMSO containing 10 mM PBS buffer at pH 7.4. Excitation was set at 383 nm. (c) The corresponding photographs of **CSA** with a UV lamp at 365 nm.

Study of β-lactamase activities in solution

The fluorescence of **DNBS-CSA** in the absence and presence of the thiol containing intermediate product **2**, was first studied to prove our design principle above (Scheme 1). As shown in Fig. 2a, **DNBS-CSA** (150 μM) displayed no fluorescence in PBS buffer (10 mM, pH 7.4), suggesting successful blocking effect of hydroxyl by sulfonate group and quenching effect by nitro groups. Upon addition of **2**, the fluorescence of **DNBS-CSA** enhanced gradually until strong yellow fluorescence (558 nm) was observed. The time-dependent fluorescence intensity change of **DNBS-CSA** was recorded without and with the addition of **2** (Fig. 2b), showing that the fluorescent light-up response only occurs in the presence of **2**.

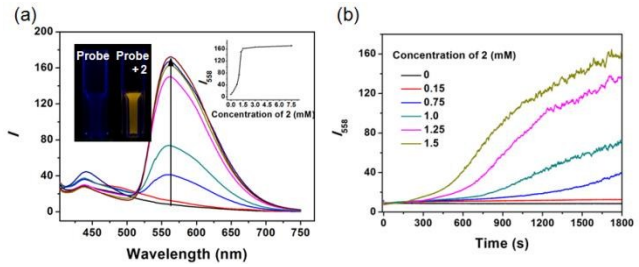

Fig. 2 (a) Fluorescence spectra of **DNBS-CSA** (150 μM) in the presence of various concentrations of **2** (0-7.5 mM) for 30 min in PBS buffer solution (10 mM, pH 7.4, 37 ℃). Insets from left to right: photographs of **DNBS-CSA** (150 μM) without or with **2** (7.5 mM) under UV light (365 nm); the corresponding fluorescence intensity (I_{558}) change over concentration of **2**. (b) Fluorescence intensity (I_{558}) change over time.

Then, the ability of **DNBS-CSA** as a sensor for β-lactamase detection was examined. As can be seen in Fig. 3, the addition of β-lactamase induced a gradual fluorescence intensity enhancement at 558 nm. In order to enable fast detection, we recorded the fluorescence intensity (I_{558}) change upon addition of β-lactamase for 8 min. It was found that the increasement rate of I_{558} within 5-8 min had a good linear relationship with the β-lactamase concentration ranging from 0 to 10 mU/mL. The detection limit was calculated as 0.5 mU/mL based on the definition by IUPAC (C_{DL} = $3S_b$/m) from 10 blank samples. This analytical performance could match up with the previously reported β-lactamase probes based on direct detection approach (see comparison of analytical performance in Table S1 in the ESI.†).

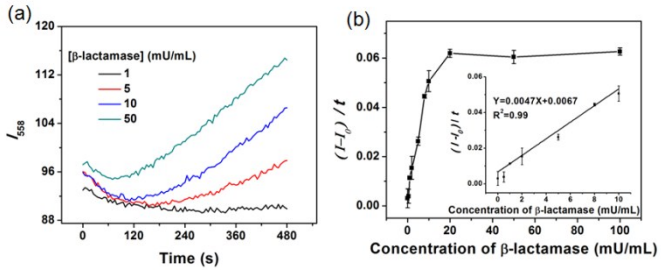

Fig. 3 Fluorescence intensity (I_{558}) change of **DNBS-CSA** (150 μM) in the presence of various concentrations of β-lactamase (0-100 mU/mL) in PBS solution containing cefazolin sodium (4.8 mM). (a) Time dependence; (b) β-lactamase concentration dependence, inset: calibration curve of I_{558} increasement rate within 5-8 min versus β-lactamase concentrations. The I_{588} increasement rate is calculated by the formula $(I-I_0)/t$, where I and I_0 are the I_{558} value at 5 min (300 s) and 8 min (480 s), respectively, while t equals to 180 s.

It has been reported by Tsien's group[1] and others[2] that β-lactam can be cleaved by β-lactamase to afford a secondary amino group. As for cephalosporin, this secondary amino group, could further trigger spontaneous elimination of any leaving group previously attached to the 3' position. On the other hand, the reaction between sulfonate and thiol to release hydroxyl has been reported by Maeda's group[53] and others[50, 54]. To verify the proposed mechanism in Scheme 1, we conducted three confirmation experiments, respectively: firstly, the formation of **2** by cefazolin and β-lactamase was confirmed (Fig. S1 in the ESI.†); secondly, the release of **CSA** by probe **DNBS-CSA** and **2** was confirmed (Fig. S2 in the ESI.†). And finally, for the mixture of cefazolin, β-lactamase and **DNBS-CSA,** the result of mass spectrum clearly suggested the formation of **CSA** (Fig. S3 in the ESI.†), supporting the hypothesis in our design (Scheme 1).

Study of β-lactamase activities on test papers

Because of the AIE characteristic of **CSA**, we further investigated the possibility of using **DNBS-CSA** as a portable test paper sensor for the detection of β-lactamase. Test papers were fabricated by dropping 5 μL solution of **DNBS-CSA** in THF (10mM) on the filter paper strips. After evaporation of THF, test papers were dipped into the sample solution containing cefazolin (4.8 mM) and different concentration of β-lactamase (0-7.0 mU/mL) for 20 min. Owing to the AIE characteristic of **CSA**, the fluorescence change of

DNBS-CSA towards β-lactamase was clearly observed by naked eyes (Fig. 4a). Image processing software Image J was used to read the intensity of fluorescent spots (Fig. S4 in the ESI.†). As can been seen in Fig. 4c, a good linearity could be found in the β-lactamase concentration range of 0-2.0 mU/mL.

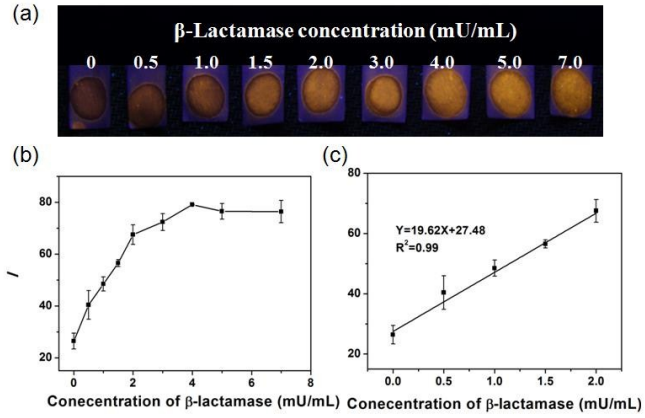

Fig. 4 (a) Photographs of test papers under a UV lamp (365 nm) for the detection of β-lactamase at various concentrations (0-7.0 mU/mL) in PBS solution containing cefazolin sodium (4.8 mM). (b) The corresponding fluorescence intensity of spots read by Image J software versus the concentrations of β-lactamase. (c) Calibration curve for β-lactamase detection.

The selectivity of **DNBS-CSA** towards β-lactamase was also studied (Fig. S5 in the ESI.†). Upon the addition of β-lactamase, the fluorescence intensity of **DNBS-CSA** was enhanced. However, almost no fluorescence intensity change was observed for some commonly used inorganic salts or biomolecules, such as $Mg(NO_3)_2$, $CaCl_2$, vitamin C, lysozyme and esterase. Thus, **DNBS-CSA** shows good selectivity to β-lactamase.

Table 1. Determination of β-lactamase in sample solution containing 10% milk.

Sample	β-lactamase Added (mU/mL)	Found (mU/mL)	Recovery (%)	R. S. D. (%)
1	0.0	0.1		6.5
2	0.5	0.5	80%	5.1
3	1.0	0.9	82%	8.4
4	1.5	1.3	81%	9.9
5	2.0	1.9	90%	6.9

The use of **DNBS-CSA** for detection of β-lactamase in milk was also investigated. Becasue of the interference from the strong abosorption of milk, the detection was conducted in solution containing 10% milk. As shown in Table 1, the results showed good recoveries and standard deviation values, suggesting **DNBS-CSA** could find potential use for β-lactamase detection in milk sample.

Conclusions

In summary, we have proposed a simple design of fluorescent probe for indirect detetction of β-lactamase activity based on AIE and ESIPT processes. Upon the addition of β-lactamase, the indirect detection approach with three-step reactions could be initiated. The intermdiate thiol containing product of cefazolin and β-lactamase could furhter react with probe **DNBS-CSA**, thus recovering ESIPT and strong fluorescence in the aggregate and solid states. Unlike other β-lactamase probes require complicated covalent modification of substrate (β-lactam ring) to probe, **DNBS-CSA** responds to β-lactamase in an indirect approach, making the modification of β-lactam ring no longer necessary. Moreover, thanks to the AIE characteristic of **CSA**, the light-up detection of β-lactamase was achieved both in aqueous solution and on test papers. For enzymes which are difficult to develop "direct approach" probes due to the complicated structures of substrates, this "indirect approach" probe design strategy provides opportunities by detecting a simpler intermediate product.

Conflicts of interest

There are no conflicts to declare.

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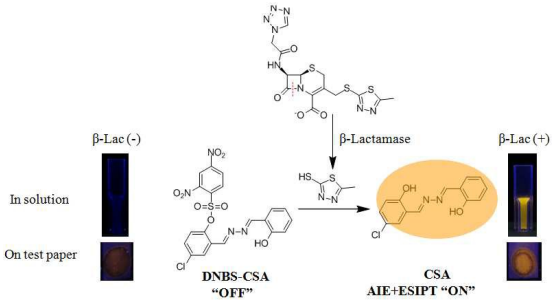
Table of contents entry



A fluorescent probe with both AIE and ESIPT characteristics has been developed for β-lactamase based on an indirect approach.