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A Switch-On Fluorescence Assay for Bacterial β-Lactamases with Amyloid Fibrils as Fluorescence Enhancer and Visual Tool

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Abstract: Herein is described the development of a novel switch-on fluorescence assay for detecting β-lactamases. The fluorescence assay comprises two components: solid beads coated with a β-lactam antibiotic, which is linked to an environment-sensitive fluorophore (dansylaminothiophenol, DTA), and amyloid fibrils of hen lysozyme (acting as fluorescence enhancer and visual tool). In the presence of the clinically significant TEM-1 β-lactamase, the DTA–antibiotic complex on the solid beads is hydrolyzed, thus releasing the DTA dye into solution. The DTA dye is only weakly fluorescent in solution but gives strong green fluorescence upon binding to lysozyme fibrils. These strongly fluorescent DTA-bound fibrils can be easily visualized by the naked eye upon illumination of the sample with a simple UV lamp. The fluorescence assay can detect TEM-1 at low concentration (0.01 nM). In contrast, no observable fluorescence appears when the fluorescence assay is performed on samples without the TEM-1 β-lactamase.

Keywords: amyloid fibrils · antibiotics · bacteria · fluorescence · sensors

Introduction

β-Lactam antibiotics have been widely used as antibacterial agents over the past several decades. These drugs function by inactivating penicillin-binding proteins (PBPs), which are responsible for synthesizing bacterial cell walls.[1] Despite this effective antibacterial function, the clinical utility of many β-lactam antibiotics has been compromised by the increasing emergence of β-lactamases, which are produced by bacteria to destroy β-lactam antibiotics. These bacterial enzymes can catalyze the hydrolysis of the cyclic amide bond in the β-lactam ring with high efficiency, thus making the antibiotic unable to inactivate PBPs.[1] β-Lactamases represent a significant indicator of antibiotic resistance in the bacterial world. The ability to produce β-lactamases has been widespread among bacteria due to wide dissemination of the genes of these enzymes.[2] As a result, β-lactamases become ubiquitous in a variety of environments, ranging from the community to the natural environment. Thus, β-lactamase monitoring becomes an important task in various areas (e.g. clinical diagnosis).

The conventional method for detecting β-lactamases involves the monitoring of bacterial growth on antibiotic-containing agar plates.[3] The spectrophotometric method has been used to monitor the hydrolytic activity of β-lactamases towards β-lactam antibiotics.[4] Detection of β-lactamases can also be performed by applying the polymerase chain reaction (PCR)[5] and the fluorescent spot test.[6] In addition, electrochemiluminescence (ECL) has been applied to detect β-lactamases.[7] Advanced analytical methods such as fluorogenic β-lactam substrates,[8,9] hydrogelation,[10] and colorimetric assay[11] have also been developed in recent years. Recently, we have developed a microplate-based method for detecting β-lactamases[12] based on a fluorescent biosensor.[13] Many of these studies have successfully demonstrated the use of advanced instrumental techniques in the detection of β-lactamases. Many advanced instrumental approaches, however, require skilled personnel and therefore limit their applicability in real applications. To facilitate the monitoring of β-lactamases, it is highly desirable to develop a 'user-friendly' analytical platform for routine applications (e.g. clinical diagnosis).

Herein, we describe the development of a 'switch-on' fluorescence assay for detecting bacterial β-lactamases. This

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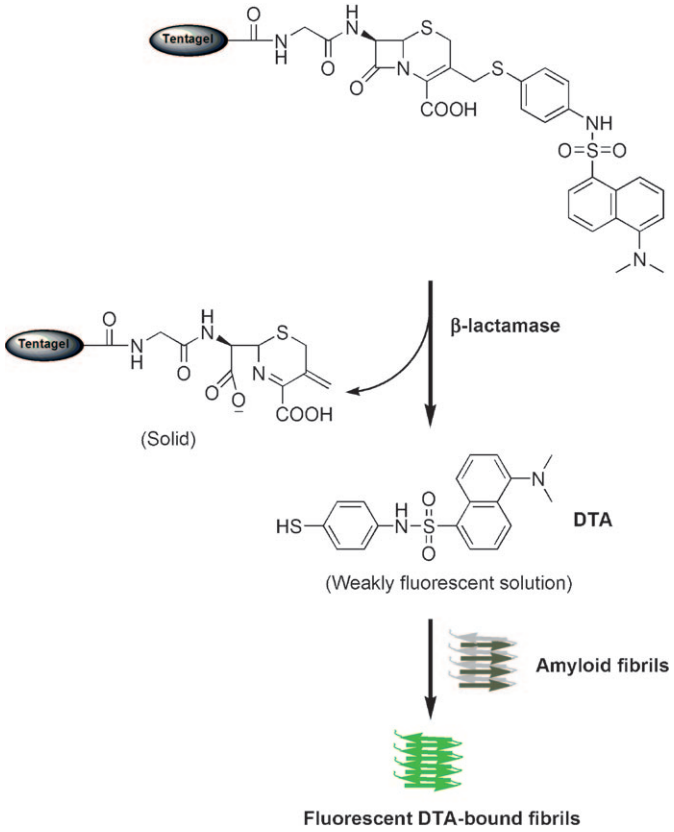
novel assay represents a general analytical platform for the biosensing of β-lactamases. The fluorescence assay comprises two components: a) solid beads coated with a fluorophore-conjugated β-lactam antibiotic and b) amyloid fibrils (acting as fluorescence enhancer and visual tool). This assay is simple in operation and allows samples with β-lactamases to be easily identified by visual observation using a simple UV lamp.

Results and Discussion

The working principle of the fluorescence assay is schematically described in Scheme 1. The assay involves the use of solid PEG–polystyrene beads (Tentagel) coated with a β-lactam antibiotic, which is covalently linked to an environment-sensitive fluorophore (dansylaminothiophenol, DTA) (Scheme 1). The solid beads are responsible for anchoring the DTA–antibiotic complex, which serves as an indicator of β-lactamase activity through the release of the DTA dye into solution. In the presence of β-lactamase, the DTA–antibiotic complex on the beads will be hydrolyzed by the enzyme, resulting in the opening of the β-lactam ring. This hydrolytic reaction will lead to the cleavage of the thioether bond at the 3′ position and subsequently release the DTA dye from the beads into solution. The solution fraction (containing the weakly fluorescent DTA dye) is then collected and mixed with amyloid fibrils of hen lysozyme (which are insoluble protein aggregates containing a highly ordered cross-β structure).[14] The fibrils will absorb the DTA dye and trigger its fluorescence strongly. In the absence of β-lactamase, there will be no β-lactam hydrolysis and therefore the DTA dye will still be covalently linked to the antibiotic on the solid beads (rather than staying in the solution). As a result, the fibrils will not receive DTA from the solution and remain non-fluorescent. With this assay design, β-lactamase can be sensitively detected based on the resulting strong fluorescence enhancement.



Scheme 1. The working principle of the fluorescence assay.

The ability of the fluorescence assay to detect the clinically significant TEM-1 β-lactamase[15] was investigated by fluorescence spectroscopy. Briefly, the solid beads coated with the DTA–antibiotic complex (ca. 100% loading, see the Supporting Information) were first incubated with TEM-1, and the solution fraction was collected and then mixed with lysozyme fibrils (prepared by incubation of hen lysozyme in 3 M guanidine hydrochloride at pH 6.3 and 50 °C;[16] Figure 1). Fluorescence measurements were then performed on this aqueous mixture. As shown in Figure 2a, a strong fluorescence peak (at 514 nm) appears after incubating the DTA–antibiotic beads with TEM-1 for 30 min. For comparison, the fluorescence assay was also performed without TEM-1. Under this condition, no significant fluorescence peak is exhibited by the assay (Figure 2a). Time-course fluorescence measurements were also performed on samples with and without TEM-1. In the presence of TEM-1, the fluorescence signal at 514 nm increases as a function of time and then becomes sustained (Figure 2b). In contrast, no significant fluorescence is exhibited by the fluorescence assay in the absence of TEM-1 (Figure 2b). These observations indicate that the assay can detect TEM-1 by giving stronger fluorescence. The ability of lysozyme fibrils to act as fluores-

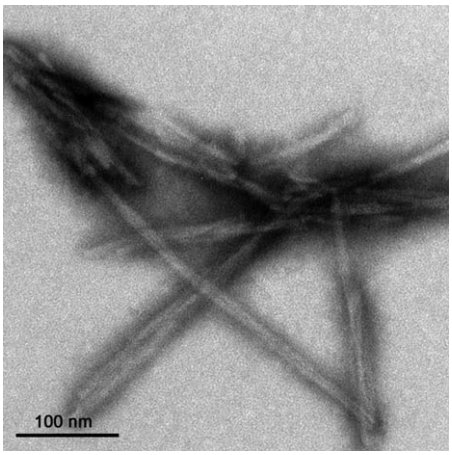


Figure 1. Transmission electron microscopy image of hen lysozyme fibrils. The fibrils were prepared by incubating hen lysozyme in 3 M guanidine hydrochloride (pH 6.3) at 50 °C. Scale bar: 100 nm.

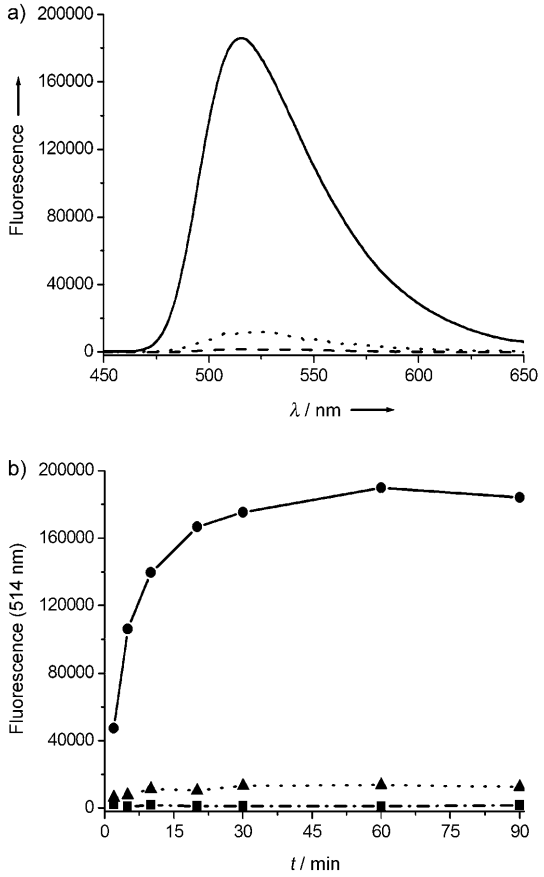


Figure 2. Detection of the TEM-1 β-lactamase by the fluorescence assay. a) Fluorescence spectra obtained with TEM-1 (—) and without TEM-1 (- -), lysozyme fibrils were used as fluorescence enhancer in both cases; fluorescence spectrum obtained with TEM-1 in the absence of lysozyme fibrils (···); assay time: 30 min. b) Time-course fluorescence signals at 514 nm recorded with TEM-1 (●) and without TEM-1(■), lysozyme fibrils were used as fluorescence enhancer in both cases; fluorescence signals at 514 nm recorded with TEM-1 in the absence of lysozyme fibrils (▲). Excitation: 350 nm; [TEM-1] = 50 nM. The fluorescence signals were subtracted from those of fibrils.

cence enhancer in the assay was also investigated. To this end, the fluorescence assay was conducted on a sample with TEM-1 in the absence and presence of lysozyme fibrils. Without using lysozyme fibrils in the assay, a weak fluorescence peak (at 525 nm) appears after incubation with TEM-1 for 30 min (Figure 2a). Interestingly, a much stronger fluorescence peak at a shorter wavelength (514 nm) appears when using lysozyme fibrils in the assay (Figure 2a); the fluorescence signal is about 13-fold larger when applying lysozyme fibrils as fluorescence enhancer in the assay. Time-course fluorescence measurements were also performed on the TEM-1 sample with and without lysozyme fibrils. As shown in Figure 2b, the fluorescence signal increases much more significantly in the presence of lysozyme fibrils over the time course, whereas the fluorescence signal increases only slightly in the absence of lysozyme fibrils. These observations highlight the ability of lysozyme fibrils to act as fluorescence enhancer in the assay.

The response of the fluorescence assay with different concentrations of TEM-1 was investigated by fluorescence spectroscopy. Figure 3 shows the time-course fluorescence pro-

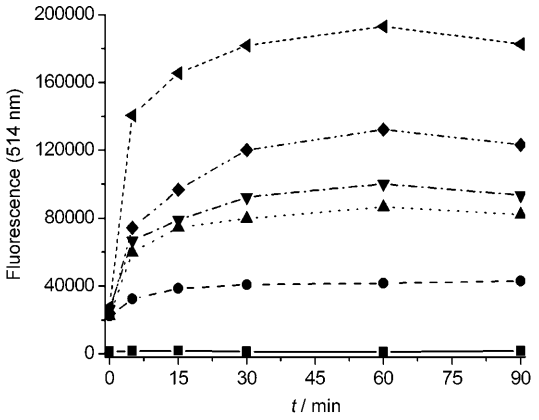


Figure 3. Fluorescence measurements of the fluorescence assay with various concentrations of the TEM-1 β-lactamase. Time-course fluorescence signals at 514 nm recorded with 0 nM TEM-1 (■), 0.01 nM TEM-1 (●), 0.1 nM TEM-1 (▲), 1 nM TEM-1 (▼), 10 nM TEM-1 (◆), and 50 nM TEM-1 (◀) in 50 mM potassium phosphate buffer (pH 7.0). Excitation: 350 nm.

files obtained with different concentrations of TEM-1. In all cases, the fluorescence signal increases as a function of time and then becomes sustained. The fluorescence signal increases more rapidly with higher concentration of TEM-1. The fluorescence assay can detect TEM-1 at subnanomolar level (0.01 nM). The sensitivity of the fluorescence assay and the conventional spectrophotometric method (with nitrocefin as the substrate) was then analyzed by comparing the analytical signals before and after enzymatic hydrolysis. As shown in Figure S1 in the Supporting Information, nitrocefin shows a stronger absorbance at 490 nm after hydrolysis by TEM-1 (50 nM); the absorbance of hydrolyzed nitrocefin increases by about 17-fold compared to that of the unhydrolyzed form. For the fluorescence assay, the fluorescence intensity increases significantly (about 174-fold larger) after the hydrolysis of the DTA–antibiotic complex on the beads by TEM-1 (50 nM) (Figure 2).

To investigate the origin of the strong fluorescence peak (at 514 nm) shown in the assay (Figure 2a), we studied the fluorescence of free DTA with lysozyme fibrils and compared it with the fluorescence result obtained from the assay (Figure 2a). As shown in Figure S2 in the Supporting Information, DTA shows a fluorescence peak (at 514 nm) with lysozyme fibrils. This observation is very similar to that shown in the fluorescence assay with TEM-1 (Figure 2a), indicating that the strong fluorescence peak (at 514 nm) shown in the assay is likely to arise from the DTA dye in the presence of lysozyme fibrils. The DTA dye is released into solution from the antibiotic on the solid beads due to β-lactam hydrolysis by TEM-1 (Scheme 1). To verify this dissociative reaction, we studied the hydrolytic reaction of the free DTA–antibiot-

ic complex with TEM-1 in solution by electrospray ionization mass spectrometry (ESI-MS). In the absence of TEM-1, a mass peak ($[M-H]^- = 626.01$), corresponding to the intact DTA–antibiotic complex (MW = 627.12), appears in the mass spectrum (see Figure S3 and Table S1 in the Supporting Information). After incubation with TEM-1 for 2 h, the mass spectrum shows a significant peak for the free DTA dye ($[M-H]^- = 357.00$; MW = 358.07) (see Figure S3 and Table S1 in the Supporting Information).

Taking the observations from the fluorescence and ESI-MS studies together, the DTA dye can dissociate from the antibiotic on the solid beads through the cleavage of the thioether bond as a result of β-lactam hydrolysis (Scheme 1).[8,10,11] The released DTA dye fluoresces only weakly at 525 nm in the aqueous solution, presumably due to the fluorescence quenching effect of water (Figure 2a). Upon addition of lysozyme fibrils, the DTA dye tends to bind to these highly ordered protein aggregates. This phenomenon can be revealed by the observation that the fibrils collected from the fluorescence assay on the TEM-1 sample exhibit green fluorescence upon illumination with the UV light (see Figure S4 in the Supporting Information). In the fibrils, the DTA dye is likely to experience a hydrophobic environment. Such an environment is revealed by the observation that the environment-sensitive fluorophore 8-anilino-1-naphthalenesulfonic acid (ANS)[17,18] shows a blue shift in emission wavelength (from 550 nm to 475 nm) and stronger fluorescence when binding to lysozyme fibrils (see Figure S5 in the Supporting Information). Upon interacting with the hydrophobic region in the fibrils, the DTA dye exhibits stronger fluorescence at a shorter wavelength (514 nm) (Figure 2a). These photophysical changes are consistent with the fact that the dansyl dye shows stronger fluorescence at a shorter wavelength when the environment becomes less polar.[18,19] As the enzymatic hydrolysis proceeds, more DTA are released from the anchored antibiotic on the beads into solution, as revealed by the MS data; the hydrolytic reaction on the DTA–antibiotic complex leads to an increasing population of free DTA and a declining population of the intact DTA–antibiotic complex (see Figure S3 in the Supporting Information). With the increasing concentration of DTA, the fibrils bind more DTA and trigger their fluorescence, thus leading to an increasing and subsequently sustained fluorescence profile over the time course (Figure 2b). In the absence of TEM-1, the DTA dye remains anchored on the solid beads rather than staying in the solution. As a result, the fibrils receive no DTA from the solution and remain non-fluorescent (Figure 2).

The ability of the fluorescence assay to detect β-lactamases in real samples was examined. In this regard, we were interested to investigate whether samples with β-lactamases can be conveniently identified by visual observation using the fluorescence assay. This simple analytical approach can be beneficial to the convenient monitoring of β-lactamases in real applications (e.g. clinical diagnosis). To examine its applicability, the fluorescence assay was performed on *E. coli* cell lysate with and without TEM-1. Briefly, the DTA–antibiotic beads were incubated with *E. coli* cell lysate, and the aqueous fractions were collected and mixed with lysozyme fibrils. The fluorescence of these samples was then monitored by illumination with a bench-top UV lamp. The bacterial cell lysate itself gives strong fluorescence (Figure 4a). This background fluorescence is so strong that it masks the green fluorescence from the DTA-bound fibrils resulting from the binding of the released DTA dye to the fibrils as a result of the hydrolysis of the antibiotic on the beads by TEM-1 (Figure 4b). This interfering effect can be, however, eliminated by using lysozyme fibrils as visual tool in the assay. The DTA-bound fibrils can be easily isolated from the fluorescent cell lysate by centrifugation. Upon illumination with a UV lamp, the collected fibrils exhibit green fluorescence (Figure 4c). This observation is similar to that shown previously (see Figure S4 in the Supporting Information), indicating that the green fluorescence arises from the binding of the released DTA dye to the fibrils as a result of the hydrolysis on the anchored antibiotic by TEM-1. For the TEM-1-free cell lysate, no observable fluorescence is, however, shown by the collected fibrils (Figure 4d). The ability of the fluorescence assay to detect TEM-1 in urine was also investigated. Similarly, green fluorescence can be observed from the fibrils collected from a urine sample with TEM-1 (see Figure S6 in the Supporting Information). These observations indicate that the fluorescence assay can conveniently identify samples with β-lactamases through simple visual observation using amyloid fibrils as visual tool.

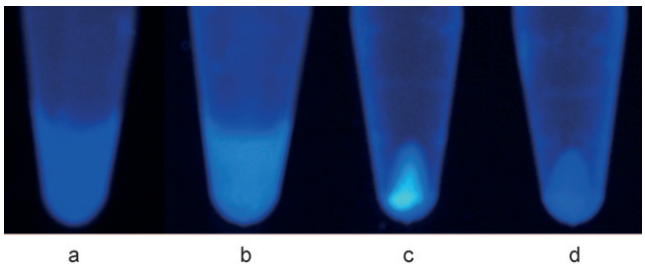


Figure 4. Detection of the TEM-1 β-lactamase in *E. coli* cell lysate by the fluorescence assay. Fluorescent images of a) *E. coli* cell lysate alone, b) cell lysate with TEM-1 treated with the fluorescence assay, lysozyme fibrils were suspended in the cell lysate, c) cell lysate with TEM-1 treated with the fluorescence assay, the lysozyme fibrils were collected by centrifugation (with the cell lysate solution discarded), and d) cell lysate without TEM-1 treated with the fluorescence assay, the lysozyme fibrils were collected by centrifugation. For (b)–(d), the cell lysate samples were incubated with the DTA–antibiotic beads for 30 min, followed by the collection of the aqueous fractions. Lysozyme fibrils were then added to these solutions. [TEM-1] = 50 nM. Excitation source: UV lamp (365 nm).

Conclusion

In this study, we have demonstrated a 'switch-on' fluorescence assay for bacterial β-lactamases. This fluorescence assay is simple in operation—it just involves mixing of samples, the DTA–antibiotic beads and amyloid fibrils. This

simple assay can detect β-lactamases by giving characteristic and strong fluorescence using amyloid fibrils as fluorescence enhancer. Moreover, with the use of amyloid fibrils as visual tool, samples with β-lactamases can be easily identified by just visual observation without using sophisticated equipment and conducting complicated analysis. This study has demonstrated the significant values of amyloid fibrils in optical biosensing development. With their useful and versatile functions, amyloid fibrils will play critical roles in the development of sensitive optical methods and visual-based sensors for bio- and chemosensing purposes in general.

Experimental Section

Experimental procedures for the synthesis of the DTA–antibiotic beads and their characterization are described in Supporting Information.

Fluorescence assays: The DTA–antibiotic beads (2 mg) were incubated with the TEM-1 β-lactamase (0.01, 0.1, 1, 10, and 50 nM) in 50 mM potassium phosphate buffer (pH 7.0, 500 μL) for different time intervals. The solution fraction was then collected and mixed with lysozyme fibrils (0.2 mg mL^{-1}). The fluorescence of the solution–fibril mixture (in a quartz cuvette with a 1 cm absorption path length) was monitored by using a FluoroLog-3 spectrofluorimeter. The excitation wavelength was 350 nm. The excitation and emission slit widths were 5 nm. The fluorescence images of the fibrils collected from the assay were recorded on a bench-top UV illuminator. For comparison, similar fluorescence experiments were also performed on samples without the TEM-1 enzyme.

Detection of TEM-1 in *E. coli* cell lysate: *E. coli* BL21(DE3) was grown in 2xTY medium (10 mL) at 37 °C with shaking at 250 rpm for 14 h. The cell pellet was collected by centrifugation and resuspended in 50 mM potassium buffer (pH 7.0, 2 mL). The cells were lysed by sonication, and the cell lysate was collected by centrifugation. The DTA–antibiotic beads (15 mg) were incubated with the cell lysate (300 μL) containing the TEM-1 β-lactamase (50 nM) for 30 min. The solution fraction was then collected and added with lysozyme fibrils (0.2 mg mL^{-1}). The fibril–solution mixture was centrifuged at 14.5k rpm for 2 min, and the solution discarded. The fluorescence image of the collected fibrils was recorded on a bench-top UV illuminator. For comparison, the fluorescence assay was also conducted on a cell lysate sample without the TEM-1 β-lactamase.

Detection of TEM-1 in urine: The DTA–antibiotic beads (15 mg) were incubated with the TEM-1 β-lactamase (50 nM) in urine (300 μL) in a microcentrifuge tube for 30 min. The solution fraction was then collected and added with lysozyme fibrils (0.2 mg mL^{-1}). The fibril–solution mixture was centrifuged at 14.5 k rpm for 2 min, and the solution discarded. The fluorescence image of the collected fibrils was recorded on a bench-top UV illuminator. For comparison, the fluorescence assay was also conducted on a urine sample without the TEM-1 β-lactamase.

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