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Enzyme-responsive reporter molecules for selective localization and fluorescence imaging of pathogenic biofilms†

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Pathogenic bacteria and their biofilm formation are responsible for a broad spectrum of microbial infections. A novel enzyme-responsive reporter molecule (ERM-1), which can specifically recognize AmpC β -lactamase (Bla) in drug resistant bacteria, has been developed to enable the selective localization of biofilms.

Recently, the emergence of antibiotic resistant bacteria has been a serious medical concern in healthcare and community care.¹ One main cause of such a rapid increase in resistance is the high-level expression of β -lactamases (Blas), a family of bacterial enzymes produced as a means of self-defence against β -lactam antibiotics including penicillins and cephalosporins, thus leading to therapeutic failure.^{2,3} As such, performing specific Blas measurements and obtaining a better understanding of their molecular mechanisms in bacterial pathogens before prescription of antibiotic therapy will be of paramount clinical importance. Among the different varieties of Blas, class A and C Blas are known as the most significant members responsible for β -lactam antibiotic resistance in bacteria. By rights, class A β -lactamases, such as, TEM-1, have been well studied for resistance inactivation and for imaging of biological processes *in vitro* and *in vivo*.⁴ Yet, as compared to these well-exploited Blas counterparts, class C Blas with similar serine hydroxyl groups in the active site have been far less investigated. Recently, class C β -lactamase genes have been found to spread worldwide and their presence leads to extensive resistance,

thus posing a remarkable clinical threat. Unfortunately, unlike the case in class A Blas, the lack of unique and selective recognition of class C Blas *in vitro* and in living systems remains a technical concern and extensive investigations still need to be further performed.

Moreover, apart from the important roles of Bla expression in antimicrobial resistance, another typical self-defence strategy for the bacterial persistence and survival from antibiotic treatment will be their modes of growth. Different from the planktonic way under most laboratory culture conditions, bacteria can easily grow as biofilms on surfaces, a type of highly populated multicellular community embedded in a biopolymer matrix, which provide bacteria additional protection against immune defenses and antibiotic treatment.⁵ Bacterial populations in biofilms usually become more resistant and thus give rise to various chronic infections that are notoriously hard to eradicate.⁵ Therefore, establishment of effective strategies to identify biofilm-associated bacterial infections will be imperative to decipher their structure and formation, as well as to facilitate the development of novel modalities for unique antimicrobial treatment.

Generally, traditional methods for biofilm identification focus on the direct visualization of their growth in a culture medium, and are usually labour intensive and time-consuming and lack the ability to enable detailed intrinsic studies for individual cells.⁶ To date, various laboratory-based methods to detect biofilm samples have been well established.⁷ Among them, optical imaging for effectively monitoring biofilm functions and biological processes has shown great potential and has been widely utilized in biomedical applications. For example, the incorporation of the green fluorescent protein (GFP) or its color variants in bacteria has been employed in studying the formation of biofilms.^{7a-c} However, the tested strains that express foreign genes may not be identical to the original bacterial pathogens. And the large size of the GFP tag (~ 27 kDa) may normally lack signal amplification, which could potentially affect the dynamics and efficiency of the whole imaging process.⁸ Moreover, several standard imaging methods based on organic fluorochromes or quantum dot nanocrystals (QDs) have also

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been utilized for visualization of the overall structure of biofilms and description of their entire expanse.^{7d-g} However, their intrinsic affinity to the bacterial biofilms may present the concerns of specificity, and meanwhile, use of fluorescent particles may also suffer from the potential issues of diffusion and toxicity.^{7,9} As such, the development of simple and specific strategies which can target biofilm structures and, more importantly, can selectively report different resistant enzymes expressed by pathogens in biofilms will be highly desirable. Unfortunately, so far such relevant studies have not been fully exploited yet.

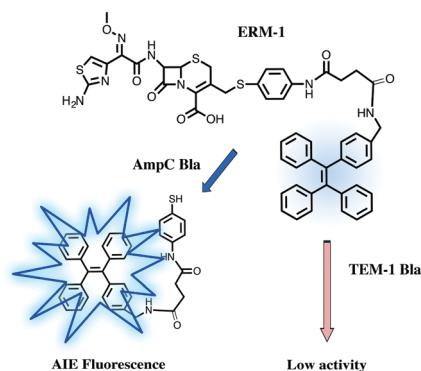
In this work, we present a unique class C AmpC Bla enzyme sensitive reporter molecule (ERM-1) that can selectively localize drug resistant pathogens in biofilms. As proof of concept, we chose a typical tetraphenylethylene (TPE) moiety as our target fluorophore, which was covalently linked to the cephalosporin structure. The major reason that we used TPE in this molecular design is mainly attributed to its promising aggregation induced emission (AIE) properties at 478 nm.⁹ Unlike the most commonly used fluorophores that may suffer from the aggregation caused fluorescence quench, these TPE based dye molecules exhibit strong emission in the aggregated state and have thus been extensively applied for biosensing and imaging in living systems.¹⁰ More importantly, the aggregated TPE products after enzyme interactions could overcome the common issues over most existing probes that may have problems of random diffusion, and can thus serve as robust fluorogenic probes to real-time image biofilms with different bacterial pathogens.

Scheme 1 illustrates the rational design and synthesis of such unique enzyme responsive reporter molecules. Typically, a 4-aminothiophenol linker was covalently introduced at the 3'-position of the cephalosporin structure, which was further conjugated with a TPE fluorophore. In order to achieve selectivity towards different Blas, one bulky methoxyimino group was connected to the 7'-amino of the β -lactam ring. Such a bulky moiety makes the cephalosporin based molecule (ERM-1) more susceptible to the AmpC enzyme, but resistant to its class A counterparts, mostly owing to its steric hindrance to block the active site in the class A enzyme pocket.¹¹ In contrast, one simple acetyl group with less steric hindrance was also introduced at the 7'-position of the cephalosporin structure to afford the controlled

reporter molecule (ERM-2). Upon the successful synthesis of the developed substrates, the final products were purified by reverse HPLC and finally characterized by NMR and mass spectrometry (ESI-MS, ESI⁺). These well-designed probe molecules will be used to react with TEM-1 and AmpC Bla enzymes, and their capability to achieve different enzyme recognitions will be systematically investigated.

The enzyme activities of reporter molecules ERM-1 and ERM-2 were first studied by measuring the fluorescence emission in phosphate buffered saline (PBS) solution (0.1 M, pH = 7.4). In the absence of Blas, there was only a small fluorescence signal observed. However, after treatment of the probes with TEM-1 and AmpC Blas separately at 37 °C for 1 h, obvious fluorescence change was detected at a wavelength of 478 nm. As shown in Fig. 1A, the maximum fluorescence enhancement in ERM-1 was ~120 fold after reaction with AmpC, whereas a decreased activity was found when ERM-1 reacted with TEM-1 and there was only ~40 fold fluorescence observed after the enzymatic reaction (Fig. 1A). These results demonstrated that enzyme hydrolysis could break the linker at the 3'-position of cephalosporins, thus resulting in the effective release of the TPE moiety. Then, the subsequent aggregation of the TPE linker leads to an enhancement in fluorescence mostly owing to the restriction of intramolecular rotation of TPE.¹⁰

Similar enzyme analysis was also carried out by using one typical AmpC inhibitor, Aztreonam (AZT).¹² The enzyme inhibition results clearly showed that AZT can greatly suppress AmpC activity. In the presence of AZT, there was little fluorescence observed after enzyme treatment (Fig. S1A, ESI⁺), clearly suggesting that developed ERM-1 can specifically recognize the AmpC enzyme. As a control, further enzymatic activity was also carried out on the basis of ERM-2 with the less bulky group at the 7'-position of cephalosporin (Fig. 1B and Fig. S1B, ESI⁺). There was ~120 fold fluorescence enhancement detected after ERM-2 was incubated with both TEM-1 and AmpC, indicating that the reporter molecule ERM-2 exhibited the same enzyme activity and it could not reflect the different enzyme recognitions between class A and C Blas. During the enzyme reaction, some non-specific fluorescence change was observed when ERM-1 was incubated with the TEM-1 enzyme. The more significant fluorescence enhancement based on ERM-1 interactions with AmpC revealed that the bulky methoxyimino group at the 7'-position of the cephalosporin structure could greatly increase the selectivity with class C Bla and thus result in a higher enzymatic reactivity.



Scheme 1 Enzyme-responsive fluorescence change upon the reaction of the reporter molecule ERM-1 with TEM-1 and AmpC Bla.

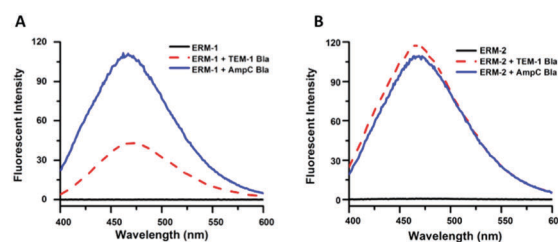


Fig. 1 (A) Emission spectra of ERM-1 and (B) ERM-2 (10 μ M) before and after incubation with TEM-1 and AmpC Blas in PBS (pH = 7.4).

Further enzyme kinetics analysis for ERM-1 and ERM-2 was carried out in PBS (pH 7.4) at 37 °C and the fluorescence changes were measured over a period of 1 h (Fig. S2, ESI†).¹³ The results demonstrated that ERM-1 could be hydrolysed by AmpC and TEM-1, respectively, with reasonable catalytic constants ($K_{\text{cat}} = 14.2$ and 3.01 min^{-1}) and Michaelis constants ($K_{\text{M}} = 11.8$ and $14.1 \text{ }\mu\text{M}$). In contrast, relevant studies were also carried out for the controlled molecule, ERM-2, and the kinetic constants for TEM-1 and AmpC were determined to be $K_{\text{M}} = 10.3$ and $11.4 \text{ }\mu\text{M}$ and $K_{\text{cat}} = 16.8$ and 15.9 min^{-1} , respectively, suggesting the promising capability of ERM-1 toward the selective recognition to AmpC enzyme reaction.

The enzyme-triggered TPE formation was further verified by HPLC and dynamic light scattering (DLS) analysis (Fig. S3 and S4, ESI†). In the presence of AmpC Bla, ERM-1 showed the complete hydrolysis of TPE with a retention time of 35 min. DLS measurements showed that the average hydrodynamic diameter of aggregated TPE was 200 nm. However, when ERM-1 reacted with TEM-1, only partial hydrolysis was found in the solution with the size distribution of the aggregated products around $\sim 100 \text{ nm}$ (Fig. S4A, ESI†). Similarly, controlled studies through the incubation of ERM-2 with AmpC and TEM-1 Blas led to the complete hydrolysis of the reporter molecule as the reaction between ERM-1 and AmpC. These results further confirmed the selective reaction of ERM-1 with the AmpC enzyme, which was consistent with the observation in the fluorescence detection.

Inspired by the results for enzyme activity in PBS solution, we investigated the applicability of ERM-1 and 2 for live cell imaging. In this study, two different Gram negative penicillin resistant bacterial strains, *Enterobacter cloacae* (*E. cloacae*) and *E. coli* BL-21, were chosen due to their high expression levels of AmpC and TEM-1 Blas, respectively.¹¹ Additionally, an antibiotic susceptible *E. coli* DH5 α strain (ATCC 53868) without Bla expression was used as a negative control. All these strains have been encoded with green fluorescent protein (GFP) plasmids, which can efficiently express the GFP and can serve as the standard for visualizing the distribution of individual bacterial pathogens. Typically, the bacterial strains were separately incubated with 20 μM of ERM-1 or 2 for 1 h at 37 °C, and subsequently subjected to confocal microscopy for fluorescence imaging. As shown in Fig. 2, strong fluorescence emission was observed after incubation of ERM-1 with AmpC expressing *E. cloacae*, whereas similar bacterial incubation with TEM-1 expressing *E. coli* BL-21 led to only weak fluorescence. Importantly, there was no obvious fluorescence detected in the control *E. coli* DH5 α bacteria and *E. cloacae* strains pretreated with the AmpC inhibitor AZT (Fig. 2A and Fig. S5, ESI†). Moreover, similar bacterial imaging experiments based on ERM-2 demonstrated obvious fluorescence in both *E. cloacae* and *E. coli* BL-21 samples. There was no fluorescence difference detected within these two strains (Fig. S6, ESI†). These results unequivocally indicated the intrinsic capability of the rationally developed enzyme responsive ERM-1 molecule to selectively report AmpC Bla activity and label the resistant bacterial pathogens. Furthermore, we explored the feasibility of quantifying the specific labelling of AmpC expressing

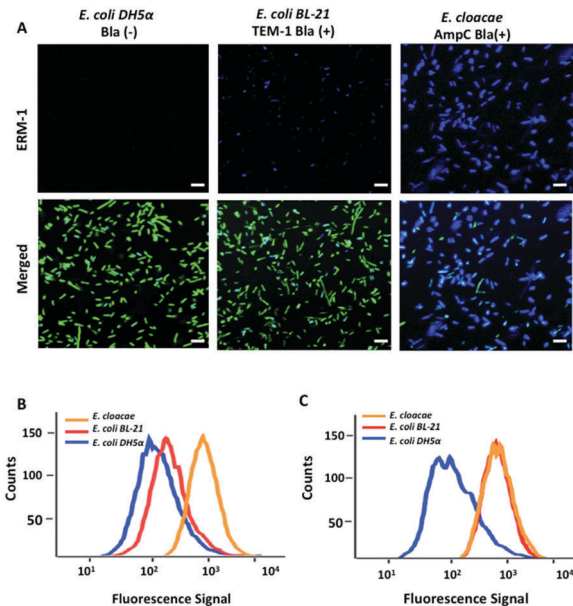


Fig. 2 (A) Confocal imaging of penicillin resistant *E. cloacae* and *E. coli* BL-21 bacteria, and antibiotic susceptible *E. coli* DH5 α strains with 20 μM of ERM-1 in 0.1 M PBS, pH = 7.4. Scale bar: 5 μm . (B) Flow cytometry analysis of ERM-1 (10 μM) with three different bacteria. (C) FCM analysis of ERM-2 (10 μM) with different bacteria.

resistant bacteria with a flow cytometer (FCM). In this experiment, three different bacterial strains (*E. cloacae*, *E. coli* BL-21, and *E. coli* DH5 α) were used to incubate with ERM-1 and ERM-2 separately (10 μM) at 37 °C for 1 h. Fluorescence signals from individual bacteria were collected at 478 nm. Fig. 2B demonstrates a strong fluorescence enhancement (~ 10 fold) for ERM-1 after incubation with AmpC expressing *E. cloacae* and a weaker fluorescence change (~ 3 fold) for TEM-1 expressing *E. coli* BL-21 as compared to the control *E. coli* DH5 α strain. Similarly, FCM studies based on ERM-2 were also carried out and the results indicated no obvious fluorescence difference between *E. cloacae* and *E. coli* BL-21 strains (Fig. 2C). These data clearly indicated that ERM-1 could serve as a reliable reporter molecule for quantifying the AmpC activity in antibiotic resistant bacteria.

Importantly, we further investigated the capability of enzyme responsive reporter molecules to selectively localize and monitor the formation of bacteria biofilms with different pathogens as models. Basically, we applied the static biofilm as a target for our study. The bacterial biofilms were cultured onto cover slips in LB broth for 24 h at 37 °C according to a protocol reported previously.⁷ During the process, the GFPs expressed in different bacteria were first used to observe the formation and distribution of individual strains within the biofilms. The biofilm structures formed by different bacterial cells were then treated with molecules ERM-1 and 2 (20 μM), respectively, for 1 h and subsequent biofilm imaging was carried out under a microscope with the excitation at 350 nm. As shown in Fig. 3, the biofilms treated with ERM-1 showed different staining patterns. There was significant fluorescence readout observed in the biofilms consisting of AmpC expressing *E. cloacae* strains, whereas only a weak signal was observed in the biofilms formed by *E. coli* BL-21, which expressed TEM-1

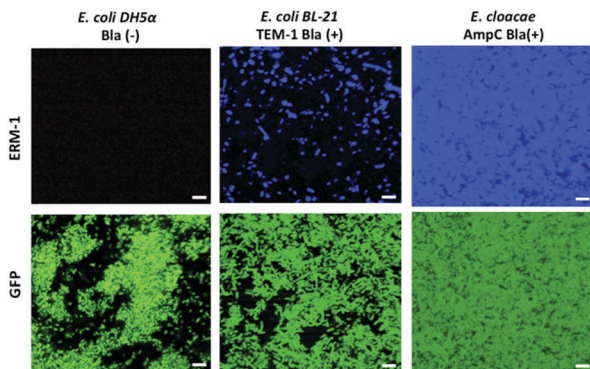


Fig. 3 Confocal imaging of penicillin resistant *E. cloacae*, *E. coli* BL-21, and antibiotic susceptible *E. coli* DH5 α bacteria biofilms with 20 μ M of ERM-1 in 0.1 M PBS, pH = 7.4. Ex = 350/50 nm; Em = 450/50 nm. Scale bar: 5 μ m.

Bla. There was no fluorescence in the *E. cloacae* biofilm in the presence of the AZT inhibitor and in the controlled biofilm with *E. coli* DH5 α (Fig. 3 and Fig. S7, ESI[†]). Although similar biofilm imaging analysis was also conducted by using ERM-2, there was no difference observed in the imaging results between the two biofilm structures with *E. cloacae* and *E. coli*, which expressed different types of bacterial antibiotic degrading enzymes (Fig. S8, ESI[†]). All these results clearly suggested that rational design of enzyme-responsive reporter molecule structures could facilitate specific targeting of biofilm components, which may thus greatly benefit the biofilm formation and controlled bacterial resistance inactivation studies.

In summary, this work presents a simple and specific approach towards the effective fluorescence imaging and localization of drug resistant AmpC β -lactamase producing bacterial strains in biofilms. By taking advantage of the bulky methoxyimino group on the 7'-position of the cephalosporin ring, selectivity recognition towards class C Bla can be easily achieved. Such enzyme responsive reporter molecules could serve as promising fluorescent probes to effectively image AmpC producing bacteria in biofilms. Importantly, this selective localization of fluorescence labelling could provide great potential for direct observation of biofilm formation from drug-resistant pathogens, and it may also provide valuable insights to benefit the effective treatment against biofilm related bacterial infections *in vitro* and *in vivo*.

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