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View Article Online
DOI: 10.1039/C6CC09296A

ChemComm

Communication

Enzyme-Responsive Reporter Molecules for Selective Localization and Fluorescent Imaging of Pathogenic Biofilms

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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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.

Currently, the emergence of antibiotics resistant bacteria has been a serious medical concern in healthcare and community[1]. One main cause of such rapid increasing of 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, and thus leading to therapeutic failure[2,3]. As such, conducting the specific Blas measurements and 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 antibiotics resistance in bacteria. By right, Class A β-lactamase, 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 this well-exploited Blas counterpart, Class C Blas with the similar serine hydroxyl group in the active site, have been far less investigated. Currently, 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, 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 Blas 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 in most laboratory culturing conditions, bacteria can easily grow as biofilms on surfaces, a type of highly populated multicellular communities 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 biofilms identification focus on the direct visualization of their growing in the culture medium, which are usually labour intensive, time-consuming and lack of detailed intrinsic studies for individual cells[6]. To date, various laboratory-based methods to detect biofilm samples have been well established[7]. Amongst them, optical imaging for effectively monitoring of biofilm functions and biological processes has shown great potentials and been widely utilized in biomedical applications. For example, the incorporation of green fluorescent protein (GFP) or its color variants in bacteria has been employed to study 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 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 (QD) have also been utilized for visualization of the overall structure of biofilm and description of their entire expanse[7d-g]. However, their intrinsic affinity to the bacterial biofilms may present the concerns of specificity, and meanwhile, usage of fluorescent particles may also suffer from the potential issue of diffusion and toxicity[7,9]. As such, development of simple and specific strategies which can target biofilm structures,

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† Electronic supplementary information (ESI) available: Synthesis and characterization of ERM-1 and ERM-2 conjugates and additional experimental details and figures. See DOI: XXXXXXXXXX

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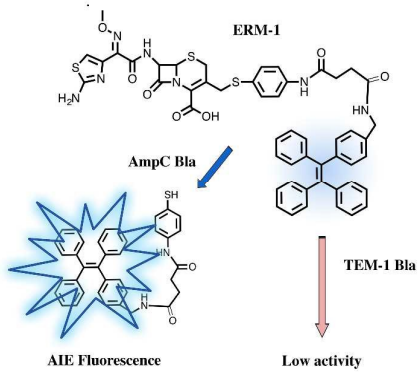
DOI: 10.1039/C6CC09296A

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 we used TPE in this molecular design is mainly attributed to its promising aggregation induced emission (AIE) property 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 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 cephalosporin structure, which was further conjugated with a TPE fluorophore. In order to achieve the selectivity towards different Blas, one bulky methoxyimino group was connected to the 7'-amino of β-lactam ring. Such bulky moiety allows the cephalosporin based molecule (ERM-1) more susceptible to AmpC enzyme, but resistant to its Class A counterparts, mostly owing to its steric hindrance to block the active site in class A enzyme pocket[11]. As contrast, one simple acetyl group with less steric hindrance was also introduced at the 7'-position of 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 Blas enzymes, and their capability to achieve



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

different enzyme recognition will be systematically investigated.

The enzyme activity of reporter molecules ERM-1 and ERM-2 were first studied by measuring the fluorescent emission in phosphate buffered saline (PBS) solution (0.1 M, pH = 7.4). In the absence of Blas, there was only little fluorescent signal observed. However, after treatment of the probes with TEM-1 and AmpC Blas separately at 37 °C for 1h, the obvious fluorescence change was detected at wavelength of 478 nm. As shown in Figure 1A, the maximum fluorescence enhancement in ERM-1 was ~ 120 folds after reaction with AmpC, whereas, a decreased activity was found when ERM-1 reacted with TEM-1 and there was only ~ 40 folds fluorescence observed after enzymatic reaction (Figure 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 TPE linker leads to an enhancement in fluorescence mostly owing to the restriction of intramolecular rotation of TPE. [10]

Similar enzyme analysis was also investigated 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 the developed ERM-1 can specifically recognize AmpC enzyme. As a control, the further enzymatic activity was also carried out on the basis of ERM-2 with the less bulky group at 7'-position of cephalosporin (Fig 1B and Fig. S1B ESI†). There was ~ 120 folds fluorescence enhancement detected after the ERM-2 incubated with both TEM-1 and AmpC, indicating that reporter molecule, ERM-2 exhibited the same enzyme activity and it could not reflect the different enzyme recognition between Class A and C Blas. During the enzyme reaction, although some non-specific fluorescence change observed when ERM-1 was incubated with TEM-1 enzyme. The more significant fluorescence enhancement based on ERM-1 interactions with AmpC revealed that the bulky methoxyimino group at the 7th position of the cephalosporin structure could greatly increase the selectivity with class C Bla and thus result in a higher enzymatic reactivity.

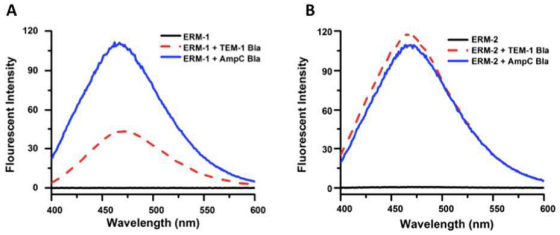


Fig. 1 (A) Emission Spectra of ERM-1 and (B) ERM-2 (10 μM) before and after incubation with TEM-1 and AmpC Blas in PBS (pH = 7.4).

The further enzyme kinetics analysis for ERM-1 and ERM-2 was carried out in PBS (pH 7.4) at 37 °C and the fluorescent changes were measured over a period of 1 hr (Fig. S2, ESI†).[13] The results demonstrated that ERM-1 could be hydrolysed by AmpC and TEM-1 respectively with the reasonable catalytic constants (K_{cat} = 14.2, 3.01 min^{-1}) and Michaelis constants (K_M = 11.8, 14.1 μM). As contrast, the relevant studies were also evaluated for the controlled molecule, ERM-2, and the kinetic constants for TEM-1 and AmpC

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were determined to be K_M = 10.3, 11.4 μM and K_{cat} = 16.8, 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 the retention time at 35 mins. DLS measurements showed that the average hydrous dynamics diameters of aggregated TPE was 200 nm. Whereas, when ERM-1 reacted with TEM-1, only partial hydrolysis was found in the solution with the size distribution of the aggregated products around ~ 100 nm (Fig. S4A, ESI†). Similarly, the 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 to 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 bacteria 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* DH5α strain (ATCC 53868) without Bla expression was used as a negative control. All these strains have been encoded with green fluorescent protein (GFP) plasmid, which can efficiently express GFP and can serve as standard for visualizing the distribution of individual bacteria pathogens. Typically, the bacterial strains were separately incubated with 20 μM of ERM-1 or 2 for 1 hr at 37°C, and subsequently subjected to the confocal microscope for fluorescence imaging. As shown in Fig. 2, strong fluorescence emission was observed after incubation of ERM-1 with AmpC expressed *E. cloacae*, whereas, the similar bacterial incubation in TEM-1 expressed *E.coli* BL-21 only led to weak fluorescence. Importantly, there was no obvious fluorescence detected in the control *E. coli* DH5α bacteria and *E. cloacae* strains pretreated with AmpC inhibitors, AZT (Fig. 2A and Fig. S5, ESI†). Moreover, similar bacterial imaging experiments based on ERM-2 demonstrated the obvious fluorescence in both *E. cloacae* and *E.coli* BL-21 samples. There was no fluorescent 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 bacteria pathogens. Furthermore, we explored the feasibility to quantify the specific labelling of AmpC expressed resistant bacteria with flow cytometer (FCM). In this experiment, three different bacteria strains: *E. cloacae and E. coli* BL-21, and *E. coli* DH5α were used to incubate with ERM-1 and ERM-2 separately (10 μM) at 37 °C for 1 hr. The fluorescence signals from individual bacteria were collected at 478 nm. Fig. 2B demonstrated a strong fluorescence enhancement (~ 10 folds) for ERM-1 after incubation with AmpC expressed *E. cloacae* and a weaker fluorescent change (~ 3 folds) for TEM-1 expressed *E. Coli* BL-21 as compared to the control *E. coli* DH5α 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.

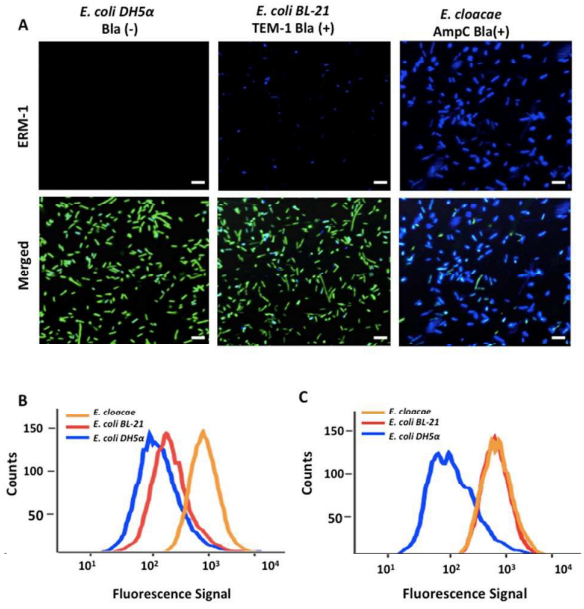

Fig. 2 (A) Confocal imaging of penicillin resistant *E. cloacae and E. coli* BL-21 bacteria, and antibiotic susceptible *E. coli* DH5α strains with 20 μM of ERM-1 in 0.1M PBS, pH = 7.4. Scale bar: 5 μm. (B) Flow cytometry analysis of ERM-1 (10 μM) with three different bacteria. (C) FCM analysis of ERM-2 (10 μM) with different bacteria.

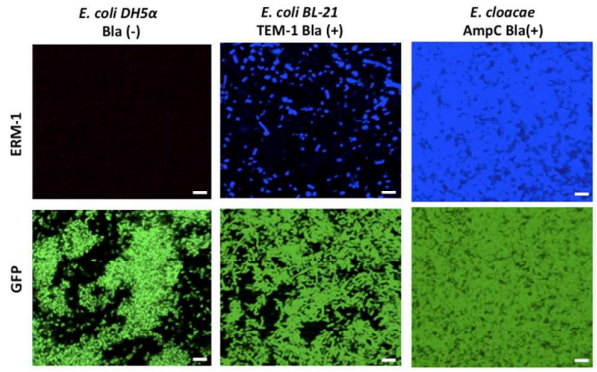

Fig. 3. Confocal imaging of penicillin resistant *E. cloacae, E. coli* BL-21, and antibiotic susceptible *E. coli* DH5α bacteria biofilms with 20 μM of ERM-1 in 0.1M PBS, pH – 7.4. Ex – 350/50 nm; Em – 450/50 nm. Scale bar: 5 μm.

Importantly, we further investigated the capability of enzyme responsive reporter molecules to selectively localize and monitor the formation of bacteria biofilm with the different pathogens as models. Basically, we applied the static biofilm as target for our study. The bacterial biofilms were cultured onto coverslips in LB Broth for 24 h at 37 °C according to the protocol reported previously[7]. During the process, the GFP expressed in different bacteria were first used to observe the formation and distribution of individual strains within the biofilms. The biofilm structures

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formed by different bacterial cells were then treated with molecules, ERM-1 and 2, (20 μM) respectively for 1 hr and subsequent biofilm imaging was carried out under microscopy with the excitation at 350 nm. As shown In Fig. 3, the biofilm treated with ERM-1 showed the different imaging staining. There was significant fluorescence readout observed in the biofilm consisting of AmpC expressed *E. cloacae* strains, whereas, only the weak signal was found in the biofilms formed by *E. coli* BL-21, which expressed TEM-1 Bla. There was no fluorescence in *E. cloacae* biofilm in the presence of AZT inhibitor and in the controlled biofilm with *E. coli* DH5α (Fig. 3 and Fig. S7, ESI†). Although the similar biofilm imaging analysis was also conducted by using ERM-2, there was no difference observed in the imaging results between 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 can facilitate specific targeting of biofilm components, which may thus greatly benefit the biofilm formation and controlled bacterial resistant inactivation studies.

In summary, this work presents a simple and specific approach towards the effective fluorescent 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 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 fluorescent labelling could provide great potential for direct observation of biofilm formation from drug resistance pathogens, it may also supply the valuable insights to benefit the effective treatment against biofilm related bacterial infections *in vitro* and *in vivo*.

The authors acknowledge the Start-Up Grant (SUG), Tier 1 RG11/13 and RG35/15 awarded by Nanyang Technological University, Singapore.

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