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Enzyme responsive luminescent ruthenium(II) cephalosporin probe for intracellular imaging and photoinactivation of antibiotics resistant bacteria†

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The Förster resonance energy transfer (FRET) based luminescent ruthenium(II) cephalosporin probe has been designed and synthesized, which can be selectively activated by endogenous β-lactamases and thus provided a localized and specific intracellular luminescence imaging and photoinactivation of drug resistant bacterial pathogens.

Since the last century, β-lactam antibiotics such as penicillins and cephalosporins have been well developed as a major class of antimicrobial agents to treat bacterial infections in clinics. However, the prevalence of β-lactam antibiotic resistant bacterial strains, especially for those of methicillin-resistant *Staphylococcus aureus* (MRSA), has emerged as a public health crisis.[1] One most remarkable mechanism of bacterial resistance is the production of endogenous β-lactamases (Blas), a type of bacterial enzymes that can effectively cleave penicillin and cephalosporin derivatives before these antibiotics target cell walls and inhibit bacterial growth.[2] As such, performing β-lactamase measurement is of clinical significance to conduct the efficient antibiotics treatment and to better understand the molecular mechanism of drug resistance, which will provide the essential knowledge on new drug development and clinical practice. Although some commonly used assays such as polymerase chain reaction (PCR), plate and culturing, event of hydrogelation and optical sensing molecules have been successfully used to identify Bla enzymes and to evaluate the bacterial therapeutic efficacy in living animals,[3] the development of simple and effective optical probes for systematic investigation of antibiotic resistant bacterial strains and thorough inactivation of drug resistant pathogens is still highly desirable and remains a challenge in the antimicrobial studies.

Recently, transition metal ruthenium(II) complexes have received considerable attention in their potential biomedical applications in luminescent sensing of DNA and oxygen species *in vitro* and in living cells, mostly attributed to their attractive photophysical properties such as long (100 ns to several ms) emission lifetime, high photostability, large Stokes shifts (above hundreds of nm) and long range of absorption.[4] More importantly, luminescent Ru(II) complexes also demonstrate potent activity to generate reactive oxygen species (*e.g.* $^1O_2$) when exposed to light of a suitable wavelength. These singlet oxygen and reactive free radicals are highly cytotoxic and can damage the cell walls and membrane structures, and thus leading to cell death.[4] As a promising alternative approach to control the microbial pathogens, the reactive oxygen species based photodynamic antimicrobial chemotherapy (PACT) has been proved effective against a variety of Gram positive and Gram negative bacteria.[5] It has been reported that some affinity ligands, such as small molecule glycopeptide antibiotics, antibodies, polypeptide cages, nanomaterials and bacteriophages, can deliver the photosensitizers (PSs) to the targeted bacterial infection sites and improve the bacterial killing efficacy towards antibiotic resistant pathogens.[6] Despite the reported effectiveness of PACT based on the target-selective delivery, indiscriminate accumulation of PSs in the available systems is still a potential limitation. The development of photoactive fluorescent imaging probes and lethal photosensitizing agents which provide precise control and selective production of $^1O_2$ upon specific bacterial enzyme reactions is necessary and needs to be further exploited.

In this study, we present a simple and effective FRET based Ru(II) cephalosporin probe for its multifunctions in selective luminescence imaging and targeted photodynamic inactivation of antibiotic resistant bacteria at the local site of infection. Scheme 1 represents the design principle of a luminescent Ru(II) cephalosporin probe (BLRu) for bacteria imaging and photoinactivation. Typically, a 4-mercaptophenyl acetic acid linker was covalently introduced at the 3′-position of cephalosporin structure and further conjugated with a luminescent $Ru(II)(bpy)_3^{2+}$ complex with a 645 nm emission maximum. The non-fluorescent quencher BHQ3[7,8] with broad absorption from 550 nm to 700 nm was connected to the 7′-amino of the cephalosporin structure through a glycyl linkage and formed the FRET pair with the $Ru(II)(bpy)_3^{2+}$ complex. The final product was purified by reverse phase HPLC and characterized by NMR and mass spectrometry (ESI-MS).

The enzyme cleavage of BLRu was studied by measuring the luminescent emission in phosphate buffered saline (PBS) solution (0.1 M, pH = 7.2). In the absence of Bla, there was only little luminescence signal mostly due to the effective FRET between $Ru(II)(bpy)_3^{2+}$ and BHQ3 moieties. However,

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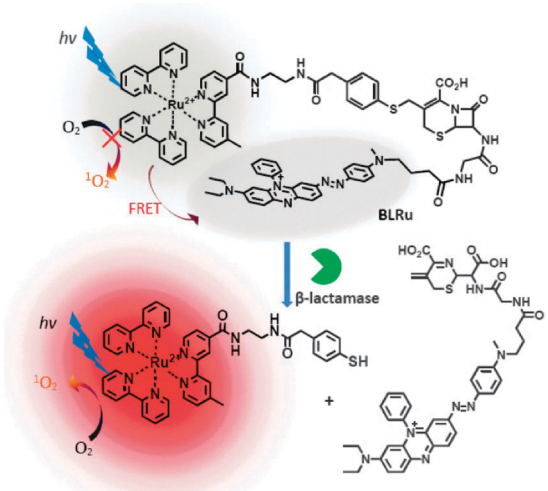

Scheme 1 Generation of the luminescent emission and photosensitivity of Ru(II) cephalosporin molecule in response to β-lactamase.

upon treatment with TEM-1 Bla at 37 °C for 2 h, there was about 8-fold emission enhancement detected at 645 nm (Fig. 1A), demonstrating that BLRu was cleaved by Bla and the enzymatic hydrolysis disrupted FRET quenching status and consequently facilitated the release of the luminescent Ru(II) complex from the conjugated cephalosporin structure (Scheme 1), which was further confirmed by HPLC analysis (Fig. S1, ESI†). Similarly, the close proximity of BHQ3 could also effectively quench the generation of $^1O_2$ from the $Ru(II)(bpy)_3^{2+}$ complex through the process of energy transfer or scavenging of produced singlet oxygen (Fig. 1B).[8] Upon Bla treatment, an obvious increase in $^1O_2$ productivity was observed by using a 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) assay[6e] and the partially recovered $^1O_2$ production was mostly due to the slow enzyme hydrolysis or the consumption of generated $^1O_2$ by the thiophenol group in the released Ru-containing fragment.[9] Further analysis of BLRu enzyme kinetics was studied in PBS (pH 7.2) at 37 °C and the results displayed that BLRu could be hydrolyzed by Bla with a catalytic constant (k_{cat}) of 16.5 ± 1.1 min^{-1} and a Michaelis constant (K_M) of 35.5 ± 2.7 μM (Fig. 1A, inset). Meanwhile, this BLRu luminescent probe was quite stable in PBS solution with the rate constant of spontaneous hydrolysis of 5.4×10^{-4} min^{-1}, which could provide a reliable enzymatic assay with the detection limit of Bla as low as 0.085 nM (Fig. S2, ESI†).

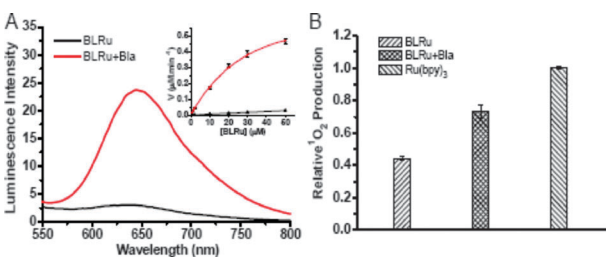

Fig. 1 (A) Emission spectra of BLRu (10 μM) before and after incubation with Bla (PBS, pH 7.2). Ex = 450 nm. Inset shows the enzymatic kinetics of TEM-1 Bla (50 nM) activity with BLRu. (B) $^1O_2$ production of BLRu (5 μM) before and after enzyme reaction by measuring ABDA (30 μM) fluorescence using $Ru(bpy)_3$ as reference.

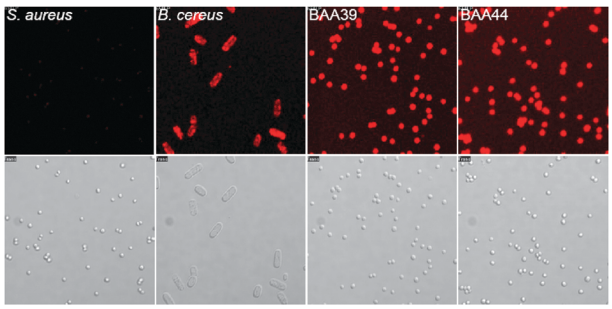

Fig. 2 Confocal microscopic (top) and differential interference contrast (bottom) images of bacteria incubated with BLRu (10 μM).

Encouraged by the information of enzyme kinetics, we investigated the applicability of BLRu to image and inactivate the β-lactam antibiotic resistant bacterial pathogens. In this study, four strains including penicillin G resistant *B. cereus* (ATCC 13061), two clinical isolates of MRSA strains (ATCC BAA39 and ATCC BAA44) and one penicillin G susceptible *S. aureus* (non-MRSA, ATCC 29213) were chosen as our main targets due to their different properties to express Blas. Wild type *E. coli* DH5α was selected as a negative control as there is no Bla expression in this bacterial strain. All these bacteria indicated the different susceptibility to penicillin G and amoxicillin but exhibited the potent activities to resist BLRu as illustrated in the minimum inhibition concentration (MIC) study (Table S1, ESI†). In order to evaluate the applicability of BLRu to image Bla activities in bacteria, living cell imaging was conducted under a confocal microscope upon laser excitation at 488 nm. As shown in Fig. 2 and Fig. S3 (ESI†), after incubation with BLRu, the confocal microscope scanning indicated the strongly increased luminescence in the penicillin resistant strains including *B. cereus* and two MRSAs. In contrast, there was much less luminescence signal detected in *S. aureus* and control *E. coli* DH5α (Fig. S4, ESI†) mostly because there was no obvious Bla expression in these bacterial strains.[10] Moreover, the sonicated bacterial lysates further confirmed the emission property of BLRu towards Bla hydrolysis in different bacterial strains, which exhibited the similar trends of Bla activities as observed in fluorescent imaging (Fig. S5, ESI†). These results clearly demonstrated the native capability of BLRu to recognize Bla in the drug resistant bacteria.

The antimicrobial activity of BLRu toward the antibiotic resistant strains through photoinactivation was also examined by a traditional surface plating method.[6] In general, all the bacterial suspensions were incubated with BLRu, exposed to white light irradiation (70 mW cm^{-2}) and spread on LB agar plates. The number of formed CFU was divided by that of strains with neither BLRu treatment nor irradiation, from which survival fraction was determined. As shown in Fig. 3A, BLRu displayed effective photoinactivation to these drug resistant MRSA strains with more than 80% lethality at only 1 μM after 84 J cm^{-2} of light irradiation, while control experiments without irradiation only showed less than 10% lethality even at 10 μM (data not shown). The killing efficacy was significantly improved with higher BLRu concentration, where a decrease of 3 orders of magnitude in bacterial survival could be easily achieved with 2 μM of BLRu for MRSA

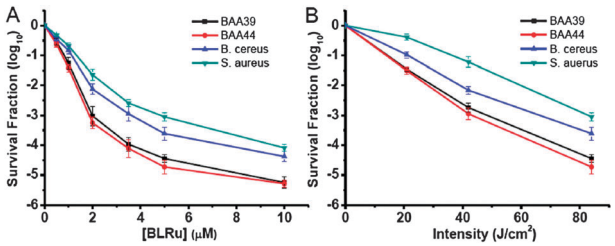

Fig. 3 Photodynamic inactivation of various bacteria at (A) different BLRu concentrations, (B) different light irradiation with BLRu (5 μM).

BAA44 and MRSA BAA39 and 5 μM of BLRu for *B. cereus*, respectively. At a lower fluence of 21 J cm^{-2}, more than 95% lethality of MRSA strains was observed in the presence of 5 μM of BLRu (Fig. 3B), which was more active than the MIC values of BLRu alone towards drug resistant bacteria, especially for those clinically isolated MRSAs. Similarly, the PACT studies indicated that penicillin G susceptible *S. aureus* strain (Fig. 3) could also be inactivated by the partially quenched $^1O_2$ production in BLRu, which illustrated the generality of luminescent BLRu for PACT studies. But compared to those penicillin G resistant *B. cereus* and MRSA strains, *S. aureus* demonstrated lower susceptibility to photoinactivation, as less $^1O_2$ could be produced due to limited Bla hydrolysis of BLRu in this cell.[10] This was also consistent with results observed in bacterial imaging studies. There was no significant photodynamic lethality observed in *E. coli* strain (Fig. S6A, ESI†) probably because of no Bla expression and the presence of different outer membrane in the cell wall architecture, which displays a lipid structure and acts as permeability barrier to prevent the effective cellular attachment of Ru(II) photosensitizers.[11] As a negative control, the photodynamic inactivation was also studied by using unmodified $Ru(bpy)_3$, which exhibited much lower lethalities to the bacterial strains as compared to those of BLRu (Fig. S6B, ESI†), mostly attributed to their different affinities toward the bacterial cell structure (Fig. S7, ESI†).[12]

In summary, this work presents an easy and effective approach for intracellular imaging and photodynamic inactivation of penicillin antibiotic resistant bacteria with high specificity and susceptibility. By taking advantage of the sensitivity of the cephalosporin core to β-lactamases and the photoactive property of the Ru(II) complex, this enzyme responsive luminescent probe could not only provide possibility to fundamentally study the mechanism of antibiotic resistance and PACT, but may also serve as promising luminescent probe to effectively image bacteria and other cells in both ensemble and single cell resolution. In addition, with more rational design, the photostable Ru(II) based luminescent molecules with higher two-photon cross section[13] and near infrared light activated photodynamic therapy properties[14] could also make them possible for biological imaging studies and potential therapeutical applications with less cellular photodamage and deeper tissue penetration in the living system.

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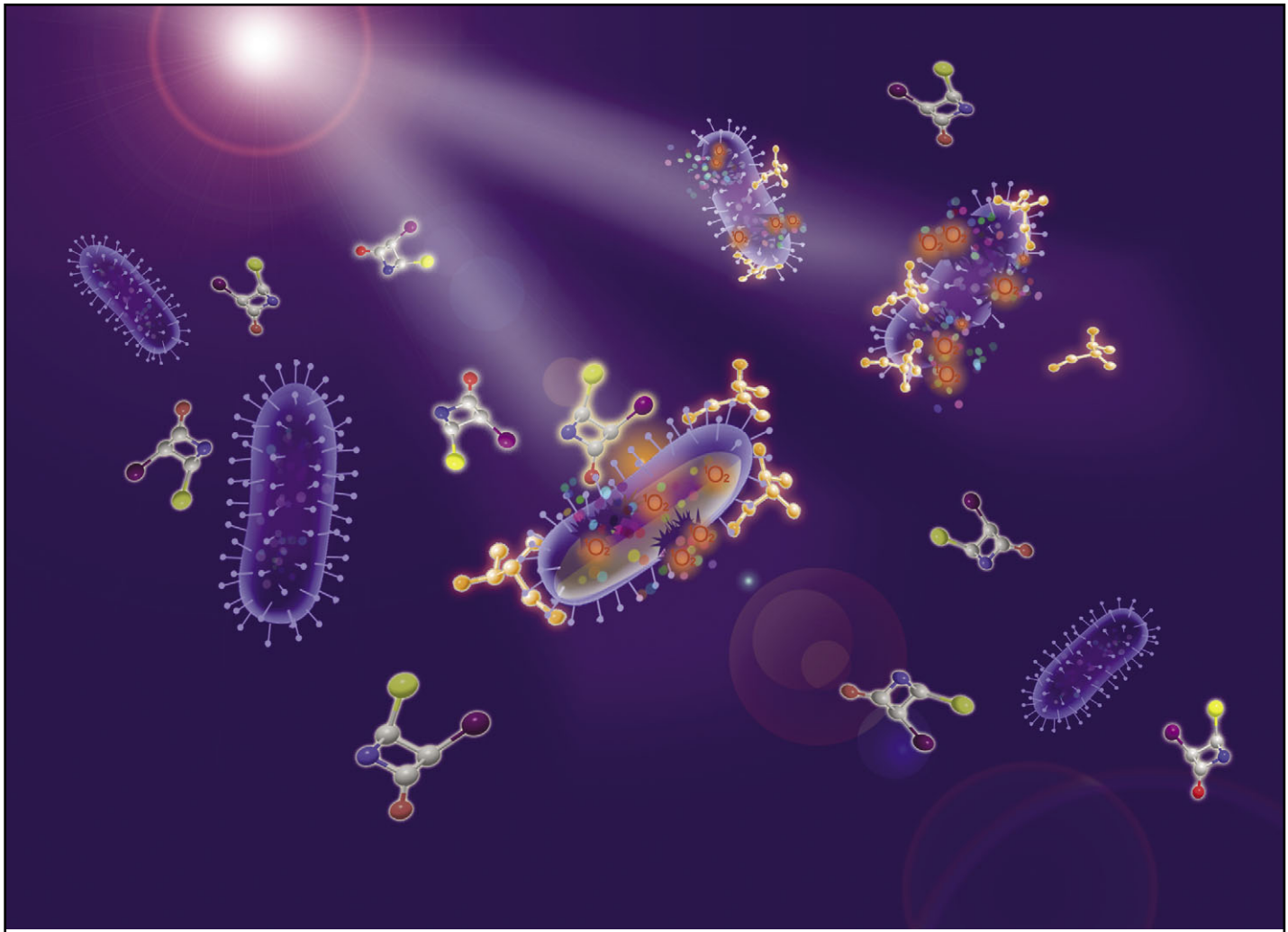

Showcasing research from the Bengang Xing lab in the Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Science at Nanyang Technological University, Singapore

Enzyme responsive luminescent ruthenium(II) cephalosporin probe for intracellular imaging and photoinactivation of antibiotics resistant bacteria

The luminescent ruthenium(II) cephalosporin probe can be selectively activated by endogenous β-lactamases, thus providing localized and specific intracellular luminescence imaging and photoinactivation of drug resistant bacterial pathogens.

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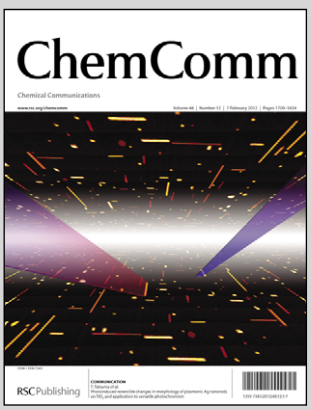

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