Construction of novel probes and their applications in resistant bacteria

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**NANYANG
TECHNOLOGICAL
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SINGAPORE

**CONSTRUCTION OF NOVEL PROBES AND
THEIR APPLICATIONS IN RESISTANT
BACTERIA**

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Abbreviations

δ	chemical shift (ppm)
Ac	acetyl
Bla	β -lactamase
Boc	<i>t</i> -butoxycarbonyl
br	broad singlet
CA	clavulanic acid
CFU	colony forming units
d	doublet
dd	doublet of doublets
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformide
fLuc	firefly luciferase
FRET	fluorescent resonance energy transfer
<i>J</i>	coupling constants
M	concentration (mol/L)
M^+	parent ion peak (mass spectrum)
m	multiplet
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
mg	milligram
MHz	megahertz
MIC	minimum inhibitory concentration

mmol	millimole
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MS	Mass spectrum
NMR	nuclear magnetic resonance
ns	nanosecond
PACT	photodynamic antimicrobial chemotherapy
PBP _s	penicillin-binding proteins
PBS	phosphate buffered saline
PS	photosensitizer
q	quarter
ROS	reactive oxygen species
RP-HPLC	reversed phase high performance liquid chromatography
rt	room temperature
Ru	ruthenium
s	singlet
t	triplet
TFA	trifluoroacetic acid
THF	tetrahydrofuran
UV-Vis	ultraviolet-visible spectroscopy
Φ	quantum yield

Abstract

The rapid spread of antibiotics resistant bacteria has raised a huge threat to our human community. The development of new techniques for the real-time visualization of such pathogenic bacteria and its biochemical changes in a human body system has become critically important. Through the rapid advancement of optical imaging technology, it has enabled us to have a non-invasive and real time monitoring for this wide spread of antibiotic resistant bacteria. One of main methods bacteria express resistance is the secretion of Beta lactamase (Bla) enzyme, which can be hydrolyzed by 4-membered beta-lactam ring in antibiotics such as penicillin leading to therapeutic failure. Therefore, it becomes increasingly important to construct novel optical imaging probes that are capable to specifically detect such resistant enzymes in living system. In this dissertation, we have synthesized and constructed some smart molecular probes to further advance their biological applications *in vitro* and *in vivo*, as shown in the following parts.

In chapter 2, a selective enzyme responsive molecule 1 termed as ERM-1 is designed and constructed to localize on the surface of pathogenic bacteria for bioimaging of the biofilm formation, which expresses AmpC, one typical class C type of beta-lactamase. In this study, we have chosen tetraphenylethylene (TPE) moiety as our target fluorophore, which was covalently conjugated to the cephalosporin molecule. One of the key reasons we used TPE is due to its promising aggregation induced emission (AIE) property at 478 nm. The aggregated TPE products after enzyme interactions could potentially overcome the common issues related with random diffusion and thus can serve as a reliable probe to real-time image biofilms with different bacterial pathogens.

In chapter 3, an enzyme responsive cephalosporin molecule conjugated lanthanide upconversion nanoparticles (UCNs) have been developed which can be used to real-time monitor antibiotic resistant pathogenic bacteria at higher wavelength with lower photo-damage and better tissue penetration. Typically, in the presence of Bla enzyme in living cells, the cephalosporin's conjugate on UCNs will be hydrolyzed at its 4-membered beta lactam ring by the serine-70 functional group by penicillin binding protein (PBP), which leads to the expulsion of the quencher Dabcyl. Thus, the FRET between FITC and Dabcyl is disrupted and enzyme activity will be detected. This specific nanoplatform can be utilized to detect β -lactamase as reporter gene and present promising imaging ability to monitor this drug resistant strains enzyme in living cells.

In chapter 4, a new study based on bacterial surface amino acid metabolism pathway has been carried out which can efficiently localize and image the enzyme substrates on bacterial pathogens for specific bacterial resistance *in vitro* and *in vivo*. Basically, N₃-contained amino acids will be involved in bacterial surface on the basis of essential metabolism pathways. Then the DBCO-contacting alkyne (C $\equiv$ C) modified beta-lactamase substrates will be covalently trapped on the bacterial surface through copper-free click chemistry. Such covalent linkage probe indicated promising activity to selectively recognize AmpC enzyme, *in vitro* and *in vivo*.

In the last chapter, a study based on an enzyme responsive probe conjugated with lipid, which can very effectively localize on the surface of the bacteria, has been developed. In this study, the cephalosporin's probe conjugated on the lipid surface

could be efficiently confined and localized in the lipid compartments of the bacteria membrane, thereby increasing the effective probe-enzyme interactions. Such unique probes indicated promising advantages compared to metabolic uptake and offers an attractive approach for labeling resistant bacteria without the need of artificial metabolic uptake or genetic engineering that may potentially perturb the endogenous properties of the bacteria.

Chapter 1

General Introduction and Latest Development

1.1 Introduction

Since the discovery of penicillin by Sir Alexander Fleming in the early 1940s for clinical application, millions of lives have been saved due to serious bacterial infections¹⁻⁴. With the initial success of antibiotics treatment, various antibiotics have been further developed in the hope of treating other forms of similar or more severe types of microbial infections^{5,6}. In general, these antibiotics drugs consist of a β -lactam ring structure, which is a 4-member ring structure that mimics the precursor of D-Alanyl-D-Alanine (D-Ala-D-Ala) present in bacteria for the final enzymatic cross-linking of bacterial cell wall biosynthesis by a penicillin-binding proteins (PBP)⁷⁻¹⁰, Figure 1.1.

Figure 1.1. Structure of the D-alanyl-D-alanine and common β -lactam ring antibiotics

Using such antibiotics that have similar backbone structure for the cross-linking process by PBP (Figure 1.2), it enables the inhibition of the biosynthesis of the cell wall and which helps to eradicate the harmful bacteria from further spreading to the human community.

Figure 1.2. The cross-linking and polymerization of peptidoglycan in bacteria cell wall biosynthesis¹

According to World Health Organization (WHO), it has been estimated that close to 10^5 to 10^6 tons of antibiotics were consumed on a yearly basis for the process of treatment. The wide spread of such usage, bacteria developed resistance against the treatment by conventional antibiotics such as penicillin¹¹⁻¹³. More importantly, there were an estimated of about 500,000 new cases of multidrug-resistant bacteria that has been identified in more than 100 countries. These high proportions of antibiotic resistance in bacteria that cause common infections such as blood stream infections, urinary tract infections etc. in all regions of the world. A substantial percentage of hospital acquired infections are caused by highly resistant bacteria such as *methicillin-resistant Staphylococcus aureus* (MRSA) and multidrug resistant bacteria that are resistant to most β -lactam antibiotics. Such resistance may lead to serious consequences, as the medical community would not be able to provide precise diagnosis and treatment to these resistant bacteria.¹⁴⁻²⁰

1.2 Consequence and Mechanism of Antibiotics Resistance Bacteria

The rapid wide spread of β -lactam antibiotics resistant bacteria may potentially cause a surge in higher death rate and caused a huge strain to the economy of the country²¹⁻³⁰. In 2016, more than 500,000 people have been affected by these pathogenic bacteria and with an estimated 30,000 people died of such infections in Europe^{53,54}. According to the reports, it has been stated that more USD 2.0 billion dollars are spent each year for bacterial infections and the amount is expected to reach 20.0 billion dollars by the year 2025^{55, 56}. Such antibiotics resistance issue will continuously remain as a global issue and bacteria will continuously evolve, mutate to battle against the use of β -lactam antibiotics which constitute 60% of worldwide antibiotics usage and render its effect⁵⁵⁻⁶⁰.

Fortunately, despite the wide spread of such resistance developed in pathogenic bacteria, government and numerous medical research institutes have recognized the potential issues related to such multi-drug resistant bacteria³¹⁻⁴⁴. Recently, intensive research has been conducted over the globe to find novel methods to accurately diagnose and treat these bacterial infection related diseases. One of the key approaches is to improve the prescription strategies and rapid read out of such resistant bacteria to prevent its further pervasive in the human community⁶¹⁻⁶⁵. According to numerous investigations that have been done, bacteria with the capability to express resistant commonly exhibit 4 different types of mechanisms (Figure 1.3): (i) The reduction of outer member proteins also known as OMPs so as to minimize the diffusion and endocytosis of β -lactam based antibiotics; (ii) The increase of efflux pumps which enables the bacteria to pump out the antibiotics that has been diffused into the bacteria membrane; (iii) The alteration of the active site of PBP so that the enzyme has lower affinity towards β -lactam based antibiotics; (iv) The formation of antibiotic resistant enzyme β -lactamase (Bla)^{1-5,66-70}. Among these different mechanisms of resistance developed by bacteria, the production of Bla is one of the most prevalent. It has been investigated that this

group of resistant enzymes (Bla) can hydrolyze the β -lactam ring which mimic the backbone structure of D-Ala-D-Ala present in most existing antibiotics before its irreversible covalent inhibition on PBP⁴⁵⁻⁵². The β -lactam drug is destroyed and render its treatment process for the patients infected with this resistant bacteria. The growing resistance in bacteria against the use of β -lactam ring based antibiotics is causing a serious threat to the health community as the use of antimicrobials has been severely compromised. In order to better understand the intrinsic nature of such enzyme, investigations have been carried out to analyze Bla which consist of 4 specific classes: A, B, C and D⁷¹⁻⁷⁵. These enzymes are generally similar in the genetic sequence (~70%) except Bla B, which is known as a metallo- β -lactamase, which makes use of a zinc metal to hydrolyze the β -lactam ring where other classes of Bla are serine based to carry out hydrolysis process. Among these 4 different classes of Bla, Class A and C have been identified as the most dominant and widespread in our health care community and research has been intensively carried out to study these groups of resistant enzymes⁸⁵⁻⁸⁸.

MECHANISMS OF ANTIMICROBIAL RESISTANCE

Figure 1.3. Types of antibiotics resistance bacteria mechanism¹

1.3 Measures to Monitor Antibiotic Resistant Bacteria

Currently, in antibiotics susceptibility methodology, one of the most common method to perform research is micro-dilutions and disk diffusion. These approaches are used to calculate the minimum inhibition concentration (MIC) to inhibit the growth of these pathogenic bacteria¹⁻¹⁰. Despite the effectiveness of such procedure, the process often requires long period of incubation and growth time, typically between 24 to 72 hours. Therefore, a more desirable method is highly recommended to rapidly detect these infectious bacteria. Other available strategies to monitor such enzymes include the use of polymerase chain reaction (PCR) or the DNA-DNA hybridization techniques. But these methods are often expensive, time consuming and may not be able to correctly provide an accurate diagnosis for the source of infections⁸¹⁻

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To address such a wide spread of bacteria with the resistant properties, there has been an increasing effort to integrate multidisciplinary approach for the successful and rapid detection of such pathogens via optical imaging techniques. With the rapid improvement made in microscopy and novel probe development, there is a rise in the investigation of these resistant bacteria through the basis of bio-imaging, which has successfully provided great insights for its fundamental nature in its tissue and cellular functions. This method has greatly aided and improved the progress of early diagnosis and preclinical investigation during the process of treatment. This process of visualization towards molecular processes and cellular events has been made possible due to the modern age of molecular imaging such as optical imaging¹¹¹, magnetic resonance imaging¹¹², positron emission tomography¹¹³ and single photon emission computed tomography (SPECT)¹¹⁴. Among these techniques, one of the most commonly used technique in optical imaging. Fluorescent imaging has attracted numerous attentions in recent decades. This is due to its high sensitivity, specificity and able to provide high spatial resolution for real-time imaging. Furthermore, fluorescent imaging also enable an accurate readout of the dynamic micro environment of these antibiotics resistant bacteria⁷⁰⁻⁷².

1.4 Recent development for imaging antibiotics resistance bacteria

Recently, many research and discoveries have been made in fluorescent imaging in antibiotics resistant bacteria. One of the most commonly used strategies to study these pathogens is involves the use of genetically engineered bacteria which expressed fluorescent proteins, for example the green fluorescent proteins. Despite the initial success to track and monitor the movement of these resistant bacteria, this method of labeling suffered from certain major drawbacks. The tagging of such large fluorescent proteins (150KDa) into these resistant bacteria would often affect the endogenous behavior of the bacteria⁵⁸⁻⁶⁰. Therefore, several effective imaging systems based on the use of nanomaterials and small molecular probes have been developed in recent years. For example, several kinds of nanoparticles (e.g., gold nanoparticles⁹³, UCNs^{96, 108}, quantum dots⁹³, carbon materials¹⁷, etc) for bacteria detection have also provided significant benefits to the biomedical field owing to their promising physical and optical properties. Comparing with the nanoparticles based probes; small molecules probes are more commonly used in optical imaging due to their higher affinity and excellent designability for bacteria pathogen-induced resistance⁷⁶⁻⁸⁴.

For example, one of the most commonly practiced strategy in the construction of small molecule probes for bacteria detection is the use of fluorescence resonance energy transfer (FRET)^{85, 93} which consist of a donor and an acceptor that have their emission and exciting wavelength overlaps effectively. Therefore, in the absent of Bla, minimum fluorescent would be observed and after Bla hydrolysis, the 4-membered beta-lactam ring would be broken and release the acceptor fluorescent by the donor molecule^{85,90-94}. This change in fluorescence

intensity allows the study of the intrinsic properties of these resistant bacteria. In order to assay the activities of Bla in resistant bacteria, many fluorogenic probes have been developed to take advantage of the high sensitivity of fluorescence detection methods. One of the most common target is the synthesis and characterization of β -lactam substrates for class A Bla, TEM-1. The design of the probes commonly based on using the phenolic fluorophores attached to the 3' position of cephalosporin moiety as leaving groups. Coumarin, fluorescein and resofurin with many other types of phenol dyes are used for the synthesis of β -lactamase substrates⁹³.

One of the most widely studied method for resistant bacteria imaging is the use of Zinc (II) dipicolylamine (Zn-DPA)¹¹⁵. This method is sensitive for tracing these pathogens as the positively charged zinc complex can electrostatically attract to the negatively charged lipopolysaccharide and carbohydrate residues located outside the outer membrane of the gram-negative bacteria. This type of interaction enables a discrimination between neutrally charged mammalian cells and negatively charged bacteria. Recently, an NIR-cyanine coupled Zn-DPA moiety was developed which demonstrated the membrane staining of bacteria cells in murine infected models (**Figure 1.4**). Furthermore, to develop Zn-DPA targeted tracers with higher quantum yield and better photostability, squaraine dye and quantum dots are applied into the study. The enhanced photostability has enabled a better real-time imaging of dividing bacteria incubated with the tracers. Despite the positive results obtained, specificity remains as a major issue with Zn-DPA probes. These moieties are able to target dead and dying cells such as in necrotic and apoptotic processes. For that reason, a more sensitive and selective probe is further developed for imaging resistant bacteria.

Figure 1.4. Fluorescence images of *S. aureus* infected mice¹¹⁵

Antimicrobial peptides (AMPs) are commonly known as short peptide sequences ranging from 12-50 residues that forms a part of the innate immune system in most classes of life. These AMPs can electrostatically adhere to the surface of the bacteria membrane and damages its integrity in the process. Although AMPs have been known for its high selectivity for its antimicrobial activities, the use of these short peptides for fluorescent studies have been limited so far¹¹⁶. For example, one of the most commonly used antimicrobial peptide for infection bio-imaging study is the use of ubiquicidin₂₉₋₄₁.¹¹⁶ Mercaptoacetyltriglycine (MAG₃) was conjugated to ubiquicidin₂₉₋₄₁ and the resulting imaging results showed a high binding to the resistant bacteria *in vivo* (**Figure 1.5**). Although AMPs have been proven to be a very effective resistant bacteria tracer, the study has been limited to only the widely studied ubiquicidin₂₉₋₄₁. Moreover, the rapid renal clearance and proteolysis of these AMPs may be a potential obstacle when a more detail study is required to carry out in living subjects. The application of AMPs for antimicrobial studies may need to be further developed before it can be used more extensively in the biomedical world.

Figure 1.5. Anterior and Posterior images of a patient injected with AMPs, ubiquicidin₂₉₋₄₁¹¹⁶

Another common method for resistant bacteria targeting is through the recognition of carbohydrate in bacteria. Carbohydrate is widely used as a building blocks for many purposes including membrane synthesis, replication virulence and as a form of energy. Therefore, carbohydrate becomes an attractive target for imaging these pathogens. For example, one of the most commonly used carbohydrate for tracing bacteria is glucose. After the conjugation of maltodextrin to the glucose molecule, IR786 was then clicked on the moiety and internalized within the bacteria by the maltodextrin transport pathway. In this way, the injected bacteria could be traced and monitored (**Figure 1.6**). Furthermore, another commonly used ligand to bind to the carbohydrate-binding protein in bacteria is cyclodextrin. After the direct labeling process, the biodistribution in living human were studied to monitor patients with knee prosthesis. Smaller analogues such as Trehalose has also been used widely to target on the surface of mycobacteria through trehalose mycolytransesterase. These enzymes are generally have a wide spectrum of substrate selectivity, therefore it is able to tolerate the bulky fluorescent molecule. Using carbohydrate as a form of selective targeting method for resistant bacteria has been a very effective strategy to monitor the development of bacterial infections and promising candidates for more clinical translation¹¹⁷.

Figure 1.6 *In vivo* imaging for *E. coli* using Maltodextrin conjugated glucose with IR786¹¹⁷

One of the most widely investigated moieties for bacterial infections is antibiotics. These drug molecules generally have very high specificity and affinity to both bacteria extracellular, intracellular proteins and enzymes. Owing to its high specificity and efficiency, antibiotics has been one of the most promising target moieties. Many fluorescent labeled antibiotics have been reported.

In the year 2011, Kazuya and co-workers successfully developed a novel genetically engineered E166N mutant of Bla that was capable to covalently labeled a FRET- quenched Bla probe for the first time⁹⁴. In this study, it has successfully demonstrated that upon Bla enzymatic interaction, there is a significant reduction in the rate of deacylation from the genetically engineered mutant species of Bla. The slower rate of deacylation in the resistant bacteria has enabled to successful labeling of the proteins of interest in living cells (**Figure 1.7**).

Figure 1.7. A genetically engineered E166N mutant form of Bla covalently labeled⁹⁴

On the same year, Leung and co-workers reported the first protein engineering of a fluorescent Amp C Bla expressed mutant for screening different classes of class C Bla inhibitors (**Figure 1.8**). The genetic engineered protein can directly detect the binding of antibiotics to its active site while maintaining its overall catalytic properties. Thus, this discovery allows the protein to function as a wild type Amp C expressed Bla and as a fluorescent drug target for in vitro drug screening¹¹⁸.

Figure 1.8. A genetically engineered Amp C expressed Bla for screening class C Bla inhibitors.¹¹⁸

In 2013, XING and co-workers develop a novel fluorogenic probe which makes use of the hydrolyzed and active quinine intermediate to perform covalent labeling in endogenous Bla enzymes³² (Figure 1.9). This elegant way of bioimaging resistant bacteria demonstrated the possibilities of labeling proteins in bacteria without undergoing any tedious and complex genetic engineering. The design of the probe is the first time demonstration of the localization of probes in endogenous resistant bacteria for the purpose of bioimaging these pathogens.

Figure 1.9 Design and synthesis of β -lactamase responsive antibiotic resistance bacterial³²

In 2014, Rao and co-workers reported the design of fluorogenic probes for specific carbapenem-resistant *Enterobacteriaceae* (CRE) and Bla expressed bacteria pathogens¹¹⁹. Carbapenemases are known as a group of β -lactamases with the ability to hydrolyze virtually all types of β -lactam based antibiotics. The rationale design of the probes is based on the fact that most carbapenem is highly resistant to Bla except carbapenemases. A close examination of the structure between carbapenem and cephalosporin reveals that one of the significant difference between the two structures is the trans stereochemistry configuration on the C6 carbon (Carbapenem) and C5 carbon (Cephalosporin). Through a simple inverted stereo chemical modification at the C5 carbon of Cephalosporin, the probe demonstrated high

selectivity towards carbapenemases and New Delhi Metallo- β - lactamase expressed bacteria (Figure 1.10).

Figure 1.10. The design of fluorogenic probes for targeting carbapenemases¹¹⁹.

In the light of this discovery for detecting carbapenemases, Xie and co-workers designed a novel probe based on the structure of carbapenem to monitor carbapenemases activities. The construction of the molecule consists of the carbapenem core structure as the enzymatic recognition moiety. Moreover, the structure is conjugated with an alkenyl-linked boron dipyrromethene (BODIPY) dye as the activatable fluorophore. Upon hydrolysis of the probe by the resistant bacteria, the compound undergoes further spontaneous degradation processes which disrupt the covalent bond between the BODIPY and carbapenem. In this way, a strong green fluorescence of BODIPY¹²⁰ is observed (Figure 1.11). In this manner, it provides as an efficient way to monitor the activities of carbapenemases in resistant bacteria through the use of carbapenem for the first time.

Figure 1.11. Design of carbapenemase-specific fluorescent probes¹²⁰

In 2015, Bachmann and co-workers successfully reported a quick and efficient way to monitor New Delhi metallo-beta-lactamase (Bla_{NDM}) through electrochemical impedance spectroscopy and fluorescent imaging¹²¹. The enhanced signal after the treatment of Bla_{NDM} specific probe with the resistant enzyme enables an efficient way to monitor such pathogenic bacteria (**Figure 1.12**).

Figure 1.12. Rapid electrochemical detection method for Bla_{NDM} ¹²¹

Bla expression is one of the commonly expressed resistant enzymes in tuberculosis (TB) in particularly Bla C. TB is one of the most infectious airborne disease caused by the wide spread of a resistant bacteria, *Mycobacterium tuberculosis* (Mtb)¹²². Hence, a rapid method to successfully monitor such diseases is of high urgency. Rao and co-workers developed a β -lactam based probe that can selectively monitor the activities of Bla C in living cells (**Figure 1.13**). The high selectivity of the probe to Bla C is achieved through the conjugation of a methoxy substitution to the 7' position of the lactam ring owing to the bigger binding pocket of Bla C. In this manner, TB diagnosis could be achieved within a shorter frame of time and allow early treatment of the diseases.

Figure 1.13 Fluorescent detection of Bla C activities¹²²

In 2016, Xie and co-workers developed a new self-immobilizing β -lactam fluorescent probe that can selectively label on the surface of the resistant bacteria and monitor its activities. The formation of such covalent bonds was confirmed by SDS-PAGE analysis and MALDI-TOF mass spectrometry¹²³. This method of labeling is useful for rapid screening of bacteria with antibiotic resistance (**Figure 1.14**).

Figure 1.14. Structures of fluorogenic probes and its labeling mechanism¹²³

However, among the different varieties of Blas, most of the existing Bla enzyme studies have been focused on Class A Bla for resistance inactivation and for imaging of biological processes. Yet, as compared to class C Blas with similar serine hydroxyl groups in the active site have been far less investigated and it has been found to rapidly spread worldwide and leads to extensive resistance against antibiotics, thus may pose a severe threat to the health community. Therefore, a more detailed and thorough investigations can be conducted to understand the behavior and properties of Class C Blas for precise treatment. Hence, there is a strong need to develop new probes that can have high selectivity and the ability to localize on Class C Bla expressed resistant bacteria for precise diagnosis⁸⁵.

Recently, Li and co-workers (**Figure 1.15**) have successfully developed a novel probe that is capable to selectively monitor a class C Bla, AmpC, which is expressed in resistant bacteria through the use of a bulky methoxyimino group conjugated on the 7th position cephalosporin's molecule compared to a smaller glycine molecule conjugated on the same position of cephalosporin's moiety. The bulky methoxyimino group provides as a source of steric hindrance to effectively block off the active site of class A Bla, *TEM-1*²⁹.

Figure 1.15 Selective detection of AmpC Bla enzyme²⁹

Moreover, apart from the important roles of Bla expression in antimicrobial resistance, another typical self-defense 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. Biofilm are formed as a result of large communities of bacteria populated together and encased by a self-generated extracellular polymeric substances (EPS). The EPS matrix serves as a protection to the bacteria within it and protects the bacteria from the invasion of antibiotics, extreme pH change and other oxidative stress. It has been researched and found that these biofilms are approximately 1000 times more resistant to antibiotic invasion than the free bacteria in the medium without the EPS as a form of protection. Furthermore, according to National Institute of Health, it has been stated that more than 80% of infections are associated with biofilm. This biofilm is capable of not only localizing on a single location but spreads rapidly and invades new territories. These invasive methods of infections by the resistant bacteria have called an immediate attention to medical researchers to rapidly find new ways to monitor such spread of biofilm infection to provide an effective treatment (**Figure 1.16**)⁹⁵

Figure 1.16. Stages of formation of biofilm matrix and its resistance against antibiotics⁹⁵

In general, traditional methods for biofilm identification focus on the direct visualization of their growth in a culture medium and are often labor intensive and time consuming. Furthermore, it lacked the ability to provide a detailed intrinsic study for individual cells^{102,103}. As such, the development of simple and specific strategies, which could successfully target these pathogens in biofilms, would be highly desirable. To date, numerous laboratory-based methods to detect biofilm samples have been well established¹⁰⁴⁻¹⁰⁷. For example, the use of DNA staining dyes such as ethidium bromide, DAPI, Syto9 that are commonly used to localize the extracellular DNA in biofilms. Furthermore, new discoveries of the use of ligand targeted ultrasound contrast agents were also being reported for the detection of biofilm under *in vitro* condition. To further improve the imaging process of biofilm to *in vivo* condition, recently, Chang and co-workers have successfully developed a novel fluorescent probe, CDy11 to monitor the activities pathogenic bacteria in biofilm in living animal. The design successfully outlines how to monitor the presence of *Pseudomonas Aeruginosa* in biofilm through confocal microscopy (**Figure 1.17**). However, the study is unable to specifically localize on class C Bla⁹⁷.

In the same year, Qu and his team found a novel method to eradicate biofilms expressed by *B. Subtilis* by releasing the caged D-amino acids on the surface of upconversion nanoparticles (UCNs) through near-infrared light irradiation. The work demonstrated the use of D-amino acids for dispersing matrix of the biofilms and hence inhibits the spread of biofilm. Despite its

initial success in application in annihilating bacteria biofilm formation, the caged D- amino acids UCNs may suffer certain drawbacks. One of the key drawback is the low quantum yield of UCNs which may potentially affect the sufficient release of D-amino acids to destroy the matrix of biofilm.

In 2016, Yang and co-workers has successfully elucidated the mechanisms for the formation of antibiotic-tolerant population in bacteria biofilms. This important discovery has enabled the biomedical researcher to provide a more efficient treatment towards biofilm-associated infections. The work was carried out using a proteomics approach in which a pulsed stable isotope labeled with the amino acids to quantify the newly expressed proteins in collistin-tolerant subpopulation of *Pseudomonas aeruginosa* biofilms¹²⁴ (Figure 1.18).

Figure 1.18. The formation of a new antibiotic-tolerant biofilm¹²⁴

Furthermore, Titz and his team developed a new strategy to inhibit lectins carbohydrate-binding proteins which showed significant reduction in the formation of biofilm. Biofilm treated with the covalent fluorescent reporter of lectins inhibitor showed a significant thinner biofilm with a reduced biomass than biofilm without the treatment of such inhibitors¹²⁵ (Figure 1.19)

Figure 1.19. Reduction of biofilm mass after binding with lectins inhibitor. A) without inhibitor and B) with inhibitor C) MALDI-TOF experiments analysis.¹²⁵

Therefore, the development of simple and specific strategies, which can selectively localize Class C Bla expressed resistant bacteria *in vitro* and *in vivo*, will be highly desirable for the detection of pathogen-induced resistance. Unfortunately, such relevant studies have not been fully investigated and the threat of Class C Bla enzyme related infections remains a major clinical concern^{89,90}.

1.5 Our Recent Designs and Developments

To overcome these challenges, in the second chapter of this thesis, a study has been carried out to examine a set of non-covalent activatable probes for rapid screening and localization of Class C Bla, AmpC, expressed resistant bacteria. These probes consist of a cephalosporin antibiotics conjugated with a unique fluorophore, tetraphenylethene (TPE), which displays an optical property that fluorescence in the aggregated state and non-fluorescent in the dispersion form. One of the key reason we used TPE is due to its promising aggregation induced emission (AIE) property at 478 nm. The aggregated TPE products after enzyme interactions may able to overcome the common issues related with random diffusion and thus can serve as a reliable probe to real-time image biofilms with different bacterial pathogens. Upon activation in the presence of AmpC Bla, the released TPE fragment would be localized on the surface of these resistant bacteria and reduce the problem associated with diffusion of the free probes which caused background interferences during confocal imaging (Figure 1.20)⁹⁸⁻¹⁰¹

Figure 1.20. Aggregation induced emission (AIE) by tetraphenylethene⁹⁶

In chapter 3, a study was carried out for the construction and application of UCNs⁹⁶ particle conjugated with β -lactam structure for monitoring the activities of gene- reporter cells expressing Bla enzyme at higher wavelength with lower photo-damage and better tissue penetration. Typically, in the presence of Bla enzyme in living cells, the cephalosporin's conjugate on UCNs will be hydrolyzed at its 4-membered beta lactam ring by the serine-70 functional group by penicillin binding protein (PBP), which leads to the expulsion of the quencher Dabcyl. Thus, the FRET between FITC and Dabcyl is disrupted and enzyme activity will be detected. This work will present the first time demonstration of using UCNs for the purpose of monitoring and detecting these resistant bacteria in cells.

In chapter 4, a study will be carried out and discuss the development of a novel metabolic probe for covalent localization of Class C Bla, AmpC, expressed bacteria for *in vitro* and *in vivo* application. Metabolic labeling provides as a powerful tool to effectively introduce chemical receptors onto the cell surface. More importantly, these externally introduced chemical functional groups can help to amplify the signals between probe-cell surfaces¹⁰⁹. After the metabolic uptake of D-Ala azide by the resistant bacteria, the bacteria will be labeled with an azide functional group on its surface. Furthermore, it is capable to undergo copper-free click ligations with a DBCO molecule conjugated with a cephalosporin's probe. The bulky DBCO group serves as a bulky side chain conjugated on the 7'position of cephalosporin's, which allows the sensitive detection of AmpC activity. After AmpC Bla activation, the quencher in the FRET pair would be released leading to change in fluorescence signal, which enables the detection and monitoring of AmpC Bla activities.

In chapter 5, a study will be discussed and illustrated on the labeling of drug resistant bacteria through an azide labeled lipid, could be efficiently confined and localized in the lipid compartments of the bacteria cell membrane, thereby increasing the effective probe-enzyme interactions¹¹⁰. Briefly, the bulky DBCO functional group on the 7th position of cephalosporin's probe helps to differentiate between Class C and A Bla. After activation of the probe by Class C Bla, it will lead to a breaking of the FRET pair which leads to a change in fluorescence intensity. Thus, compare to artificial metabolic uptake and complex genetic engineering techniques, the proposed unique probes indicated promising advantages for labeling resistant bacteria, which can be applied to biomedical applications in antibiotic resistance pathogens.

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Chapter 2

Enzyme-Responsive Reporter Molecules For Selective Localization and Fluorescence Imaging of Pathogenic Biofilms

2.1 Introduction

The rapid rise of pathogenic bacteria and its biofilm formation has been recognized to be accountable for a broad spectrum of known microbial infections and diseases. The high-level of expression for Bla, commonly known as a family bacterial enzymes, which evolve as a result of the side spread use of antibiotics has caused an alarm in the medical community for its ability to battle against β -lactam ring and render off its treatment effect¹⁻⁴. Therefore, there is a great need to find new methods that is able to selectively monitor such resistant enzymes for the precise diagnosis of bacterial infections. Blas family consist mainly 4 classes, which are known as: A, B, C and D. Among these classes of enzymes, A and C are the most dominant and responsible for a variety of microbial infection diseases. Over the past decades, many researches have been conducted towards the investigation of class A Bla, in particular TEM-1 for its resistance inactivation as well as bioimaging its biological activity for both *in vitro* and *in vivo* studies.⁷⁻¹⁰ However, comparisons to its counterpart, Class C Bla such as AmpC and P99 have not been widely understood. Although, some investigations have been conducted previously and found that it has a close relationship with TEM-1 owing to its similar serine group in the active site of the Bla enzyme which is responsible for hydrolyzing the β -lactam ring structure, its studies are still under exploited so far. The wide spread of Class C Bla worldwide is real and posed a serious threat to the human community⁵⁻⁸. The lack of a proper and selective probe for the recognition of this class C Bla for *in vitro* and *in vivo* systems remains a technical concern and the needs for more extensive investigation is critically required to be performed.

Furthermore, besides the expression of Blas by these resistant bacteria, another type of self-defense strategy by these pathogens are its mode of growth^{10,11}. Bacteria grow rapidly under laboratory culture condition and able to form layers of biofilm, which is a layer of polymer matrix that act as a form of self-defense when antibiotics is prescribed for treatment, on its surface. This biomatrix provides as a form of additional defense against the foreign agents from invading the bacteria populations. It has been investigated that the biofilm formed by this group of resistant bacteria generally has at least a 1000 times capability to resist the annihilation by antibiotics treatment¹⁰⁻²⁰. Therefore, it has become increasingly difficult to eradicate such pathogenic bacteria and its biofilm. To this end, it becomes not only important for modern research to develop novel strategy to detect such resistant bacteria, but also ways to identify biofilm-associated forms of infections for treatment.

In general, the traditional method for identifying biofilm is the use of microscopy for direct visualization of their growth within a culture medium^{13,14}. Despite, it being the widely practiced method in clinics and hospital, this process is often laborious and time consuming. On top of this, it lacks the ability to provide an intrinsic study of the individual pathogens within a biofilm matrix. To date, there are several laboratory- based methods that have been developed for the purpose of detecting biofilm samples. Among all these methods, optically imaging is one of the most commonly employed for effective monitoring of biological processes and biofilm functions in various biomedical applications²⁰. One such example is the incorporation of Green Fluorescent Protein (GFP) or its other color variants into individual bacteria has enabled researchers to study the formations of biofilms by these resistant bacteria strains. Despite its initial successes to detect these biofilm formations, the large of size of such

fluorescent proteins ($\sim 27\text{kDa}$)¹⁹ is often deficient in signal amplification, which essentially may affect the efficiency as well as the dynamics of the entire imaging process. In addition, efforts have also been made into the development of novel quantum dot nanocrystals (QDs)²¹ or organic fluorochromes for the purpose of visualization of the biofilm overall structure and the description of their entire expanse. However, the lack of affinity to the specific class of Bla expression by the pathogens in the biofilm matrix represent a major concern and problem associated with the diffusion of fluorescent particles upon activation by Bla may essentially decrease the signal to background ratio²¹⁻²⁵. Furthermore, nanoparticles such as QDs have always been associated with high level of toxicity for *in vivo* and cells studies, hence its application to the medical community would be greatly limited. As such, the development of a novel strategy that is simple and specific for the localization of AmpC express resistant bacteria and its biofilm formation is highly desirable²⁶⁻³¹.

2.2 Design and Methods

We constructed an enzyme sensitive reporter molecule (ERM-1) for the purpose of imaging and localizing drug resistant pathogens expressing AmpC Bla in biofilms (Scheme 2.1).

Scheme 2.1: Fluorescent enhancement observed after ERM-1 hydrolyzed at the β -lactam ring after the reaction with TEM-1 and AmpC Bla. TPE molecules is released and aggregated to cause induced emission.

As a proof of our concept in this particular study, tetraphenylethylene (TPE) moiety has been chosen as our target fluorophore. The TPE molecule is then covalently ligated onto the cephalosporin 3' position. One of the key reasons for our choice of fluorophore is the unique fluorescent characteristic that TPE displayed which is known as aggregation induced emission (AIE)^{11,32}. Typically, TPE molecule under non-aggregated state has its tetraphenyl rings under constant rotation, which dissipate energy to the surrounding leading it to be in a non-fluorescent state. Upon aggregation of these TPE molecules, the free rotation of these phenyl rings will be restricted and hence induce a strong emission of fluorescent. This phenomenon is advantageous to our design, as the aggregated product upon Bla activation would have this ability to localize on the surface of the bacteria without suffering the fate of diffusion. The emission properties of AIE generally takes place at 478 nm and display the strong fluorescent emitted by TPE upon aggregation³². This unique behavior is unlike most fluorophores which may suffer the common issues of aggregation caused quenching (ACQ). Due to the special characteristic by AIE, it has been widely used in biosensing as well as *in vivo* imaging.

Synthesis of ERM-1 and ERM-2

Synthesis of 2:

Diphenylmethane (2.03 g, 15 mmol) was dissolved into anhydrous THF (50 mL) and was subsequently cooled to 0°C. Next, n-butyllithium (7.50 mL, 2.5 M) was added drop wise into the reaction mixture. After stirring for 1 hr, 4-methylbenzophenone (2.45 g, 12.50 mmol) was added and allowed to slowly warm to room temperature while being stirred overnight. The reaction mixture was then cooled to room temperature and quenched with aqueous ammonium chloride. The organic materials were extracted thrice with ethyl acetate. The combined extracts

were washed thrice with water and once with brine, and dried over MgSO_4 . The solvents were removed under reduced pressure and the crude was purified by flash column chromatography (silica gel, *n*-hexane/ethyl acetate = 99:1) and is followed by purification with flash chromatography to afford **2** as a white solid (1.69 g, 4.88 mmol, yield 79%). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.08–7.21 (m, 15H), 7.04 (m, 4H), 2.37 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 144.08, 141.07, 140.90, 140.62, 136.15, 131.48, 131.44, 131.36, 128.51, 127.81, 127.75, 126.46, 126.41, 21.33. MS (ESI) m/z : 347.24. calculated for $[\text{M}+\text{H}]^+$: 347.47.

Synthesis of **3**:

Compound **2** (1.04 g, 3 mmol) was dissolved in CCl_4 (20 mL), followed by addition of *N*-bromosuccinimide (0.55 g, 3.2 mmol) and a catalytic amount of benzoyl peroxide. Reaction mixture was stirred under reflux condition for 12 h. The organic materials were extracted thrice with CH_2Cl_2 . The combined extracts were washed thrice with water and once with brine, and dried over MgSO_4 . The solvents were removed under reduced pressure and the crude was purified by flash column chromatography (silica gel, *n*-hexane/ethyl acetate = 99:1) to afford **3** as a white solid (0.71 g, 1.67 mmol, yield 61%). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.16–7.00 (m, 19H), 4.43 (s, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 145.01, 144.11, 143.74, 143.44, 141.49, 140.14, 139.09, 135.79, 131.66, 131.28, 128.51, 127.68, 126.56, 33.64. MS (ESI) m/z : 425.54, calculated for $[\text{M}]^+$: 425.37

Synthesis of 4:

Compound **3** (0.85 g, 1.67 mmol) was dissolved in dimethyl sulfoxide (10 mL), followed by addition of sodium azide (0.20 g, 3 mmol). The reaction mixture was stirred under a nitrogen atmosphere at room temperature overnight. Subsequently, mixture was quenched with water (50 mL). The organic materials were extracted thrice with diethyl ether. The combined extracts were washed thrice with water and once with brine, and dried over MgSO_4 . The solvents were removed under reduced pressure and the crude was purified by flash column chromatography (silica gel, *n*-hexane/Chloroform = 80:20) to afford **4** as a white solid (0.73 g, 1.88 mmol, yield 95%). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.08-7.17 (m, 21H), 4.30 (s, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 144.01, 143.61, 141.59, 140.39, 133.40, 131.84, 131.41, 127.87, 127.76, 127.67, 126.63, 54.59. MS (ESI) m/z : 409.64, calculated for $[\text{M}+\text{Na}]^+$: 409.49.

Synthesis of 5:

Compound **4** (0.73 g, 1.88 mmol) was dissolved in anhydrous methanol (10 mL), followed by addition of triphenylphosphine (1.00 g). The reaction mixture was stirred under reflux condition overnight. After confirmation with TLC, crude mixture was concentrated under reduced pressure and purified by flash column chromatography (silica gel, *n*-hexane/ethyl

acetate = 20:80) to afford **5** as a white solid (0.55 g, 1.52 mmol, yield 81%). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.12–7.01 (m, 19H), 3.84 (s, 2H), 1.75 (s, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 143.70, 143.54, 143.51, 141.40, 140.23, 134.61, 131.76, 131.26, 127.78, 127.72, 127.66, 127.45, 126.57, 126.53, 43.88, 23.22. MS (ESI) m/z : 361.18, calculated for $[\text{M}]^+$: 361.49

Synthesis of **6**:

Compound **5** (0.55 g, 1.52 mmol) was dissolved in CH_2Cl_2 (10 mL), followed by addition of succinic anhydride (0.15 g, 15 mmol) and *N,N*-diisopropylethylamine (261 μL). The reaction mixture was stirred at room temperature overnight. After confirmation with TLC, crude mixture was concentrated under reduced pressure and purified by flash column chromatography (silica gel, *n*-hexane/ethyl acetate = 50:50), to afford **6** as a white solid (0.31 g, 0.67 mmol, yield 45%). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.02–7.14 (m, 20H), 4.38 (d, $J = 6.00$ Hz, 2H), 2.74 (t, $J = 6.00$ Hz, 2H), 2.54 (t, $J = 6.00$ Hz, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 143.60, 143.26, 141.28, 140.32, 135.54, 131.70, 131.28, 127.75, 127.70, 127.65, 127.03, 126.50, 43.61, 30.73, 29.69. MS (ESI) m/z : 462.09, calculated for $[\text{M}+\text{H}]^+$: 462.56

Synthesis of **7**:

2-(2-amino-1,3-thiazol-4-yl)-2-(methoxyimino)acetic acid (500 mg, 4.97 mmol) was

dissolved in CHCl_3/DMF 2:1 (20 mL) and TEA (1.5 mL, 11 mmol). The reaction mixture was stirred at 0°C . Subsequently, 4-methyl trityl chloride (800 mg, 5.47 mmol) was added to the solution. The reaction mixture was slowly warmed to room temperature and stirred overnight. After confirmation with TLC, chemical mixture was quenched with aqueous hydrogen chloride (1M). The organic materials were extracted thrice with CHCl_3 . The combined extracts were washed thrice with water and once with brine, and dried over MgSO_4 . The solvents were removed under reduced pressure and the crude was purified by flash column chromatography (silica gel, *n*-hexane/ethyl acetate = 80:20) to afford **7** as a white solid (413 mg, 1.00 mmol, yield 45%). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.12-7.31 (m, 15H), 6.58 (s, 2H), 3.94 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 169.38, 141.91, 136.83, 129.38, 128.39, 127.92, 108.25, 72.94, 63.08, 29.71, 14.14. MS (ESI) m/z : 479.99, calculated for $[\text{M}+\text{Na}]^+$: 480.55

Synthesis of **8**:

7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid diphenylmethyl ester hydrochloride (413 mg, 1.00 mmol) was dissolved in CH_2Cl_2 (10 mL), followed by addition of N, N-diisopropylethylamine (174 μL), acetyl chloride (71 μL) and 2,6-lutidine (116 μL). The reaction mixture was stirred under room temperature overnight. After confirmation with TLC, organic materials were extracted thrice with CH_2Cl_2 . The combined extracts were washed thrice with water and once with brine, and dried over MgSO_4 . The solvents were removed under reduced pressure and the crude was purified by flash column chromatography (silica gel, *n*-hexane/ethyl acetate = 50:50) to afford **8** as a white solid (390 mg, 0.78 mmol, yield 85%). ^1H NMR (300 MHz, CDCl_3)

δ (ppm): 7.25-7.44 (m, 10H), 6.75 (d, $J = 8.91$ Hz, 1H), 5.90 (dd, $J = 4.92$ Hz, 8.91 Hz, 1H), 4.99 (d, $J = 4.98$ Hz, 1H), 4.40 (s, 2H), 3.53 (q, $J = 17.97$ Hz, 2H), 2.03 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 170.64, 165.44, 160.54, 139.11, 138.96, 128.66, 128.54, 128.34, 128.22, 127.71, 127.39, 126.95, 125.57, 79.89, 77.53, 77.11, 76.69, 59.20, 57.75, 43.18, 27.24, 22.74. MS (ESI) m/z : 456.87, calculated for $[\text{M}]^+$: 456.94

Synthesis of 9:

7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid diphenylmethyl ester hydrochloride (451 mg, 1 mmol) was dissolved in dimethylformamide (10 mL), followed by addition of pyridine (79.1mg, 1 mmol), EDC hydrochloride (191.7 mg, 1 mmol) and compound 7 (447 mg, 1 mmol). The mixture was stirred under room temperature overnight. After confirmation with TLC, organic materials were extracted thrice with ethyl acetate. The combined extracts were washed thrice with water and once with brine, and dried over MgSO_4 . The solvents were removed under reduced pressure and the crude was purified by flash column chromatography (silica gel, n -hexane/ethyl acetate = 50:50) to afford a yellow solid 9 (469.70 mg, 0.87 mmol, 55%). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.26-7.37 (m, 19H), 7.11-7.19 (m, 7H), 6.87 (d, $J = 8.94$ Hz, 1H), 6.73 (s, 1H), 5.96 (dd, $J = 4.92$ Hz, 8.91Hz, 1H), 5.08 (d, $J = 4.95$ Hz, 1H), 4.40 (d, $J = 2.97$ Hz, 2H), 4.04 (s, 3H), 3.56 (q, $J = 18.00$ Hz, 2H), 2.33 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 168.62, 163.97, 162.31, 160.42, 147.19, 143.40, 143.24, 140.80, 140.05, 139.05, 138.92, 137.43, 129.27, 129.21, 129.17, 128.97, 128.65, 128.57, 128.36, 128.24, 127.75, 127.55, 126.99, 125.75, 113.03, 71.58, 63.32, 58.94, 57.70, 43.10, 27.36, 21.04. MS (ESI) m/z : 854.65, calculated for $[\text{M}+\text{H}]^+$: 854.44

Synthesis of 10:

Compound **8** (250 mg, 0.50 mmol) was dissolved in dimethyl formamide (2 mL), followed by addition of 4-aminothiophenol (63 mg, 0.50 mmol). The reaction mixture was stirred under room temperature for 10 minutes. Subsequently, sodium iodide (150 mg, 1.00 mmol) and 2,6-lutidine (58 μ L) was added. The chemical mixture was stirred overnight. After confirmation with TLC, organic materials were extracted twice with ethyl acetate, water and brine. The combined extracts were then dried over reduced pressure and purified using flash chromatography to afford **10** (201 mg, 0.37 mmol, 74%). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.27-7.34 (m, 11H), 7.12 (d, $J = 6.00$ Hz, 2H), 6.89 (d, $J = 8.76$ Hz, 1H), 6.45 (d, $J = 6.00$ Hz, 2H), 5.80 (dd, $J = 4.71$ Hz, 8.85 Hz, 1H), 4.88 (d, $J = 4.71$ Hz, 1H), 4.20 (d, $J = 8.79$ Hz, 1H), 3.59 (d, $J = 13.08$ Hz, 1H), 3.36 (q, $J = 17.76$ Hz, 2H), 2.04 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 170.55, 165.32, 160.66, 147.11, 139.64, 139.42, 135.70, 133.71, 128.75, 128.12, 127.97, 127.18, 123.97, 120.57, 79.26, 59.08, 58.01, 38.91, 28.75, 22.80. MS (ESI) m/z : 545.98, calculated for $[\text{M}]^+$: 545.67

Synthesis of 11:

Compound **9** (427 mg, 0.50 mmol) was dissolved in dimethyl formamide (2 mL), followed by addition of 4-aminothiophenol (63 mg, 0.50 mmol). The reaction mixture was stirred under room temperature for 10 minutes. Subsequently, sodium iodide (150 mg, 1.00 mmol) and 2,6-lutidine (58 μ L) was added. The chemical mixture was stirred overnight. After confirmation with TLC, organic materials were extracted twice with ethyl acetate, water and brine. The combined extracts were then dried over reduced pressure and purified using flash chromatography to afford **11** (349 mg, 0.37 mmol, 74%). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.02-7.40 (m, 27H), 6.89 (s, 1H), 6.86 (d, $J = 6.00$ Hz, 2H), 6.76 (d, $J = 6.00$ Hz, 2H), 6.46 (d, $J = 6.00$ Hz, 2H), 5.89 (dd, $J = 4.68$ Hz, 8.76 Hz, 1H), 4.98 (d, $J = 4.74$ Hz, 1H), 4.13 (s, 3H), 3.67 (d, $J = 13.29$ Hz, 2H), 3.61 (s, 2H), 3.36 (d, $J = 17.80$ Hz, 1H), 2.35 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 168.62, 163.68, 162.24, 160.54, 147.22, 147.03, 143.33, 143.27, 140.03, 139.58, 139.38, 137.45, 135.66, 132.82, 129.20, 128.96, 128.54, 128.44, 128.23, 128.10, 127.96, 127.62, 127.57, 127.02, 124.16, 120.66, 115.47, 113.22, 79.22, 71.58, 63.32, 58.76, 57.80, 38.91, 28.86, 21.02. MS (ESI) m/z : 944.77, calculated for $[\text{M}]^+$: 944.17

Synthesis of ERM-1:

Compound **11** (170 mg, 0.18 mmol) was dissolved in dimethyl formamide (10 mL), followed by addition of compound **8** (81.80 mg, 0.18 mmol), ethylcarbodiimide hydrochloride (70.30 mg, 0.37 mmol) and *N*-methylmorpholine *N*-oxide (20 μ L). The reaction mixture was stirred overnight under room temperature. After confirmation by TLC, organic materials were extracted thrice with ethyl acetate. The combined extracts were washed thrice with water and once with brine, and dried over MgSO_4 . The crude mixture was subsequently added with CH_2Cl_2 (1 mL), trifluoroacetic acid (300 μ L), and anisole (20 μ L) was added into the reaction mixture, which was stirred under room temperature for 1 h. The solvents were removed under reduced pressure and subsequently crude mixture was purified by reverse phase HPLC to afford white solid **ERM-1** (38.3 mg, 22%). ^1H NMR (300 MHz, Acetone- D_6) δ (ppm): 6.91-7.31 (m, 21H), 6.75 (d, $J = 8.28$ Hz, 1H), 6.64 (d, $J = 8.49$ Hz, 2H), 5.71 (t, $J = 4.92$ Hz, 1H), 5.15 (d, $J = 4.74$, 1H), 4.35 (s, 2H), 3.88 (s, 3H), 3.94 (d, $J = 17.76$ Hz, 1H), 3.47 (d, 18.69 H), 3.47 (d, $J = 18.21$ Hz, 1H), 2.63 (t, $J = 6.30$ Hz, 2H), 2.58 (t, $J = 5.61$ Hz, 2H). ^{13}C NMR (75 MHz, Acetone- D_6) δ (ppm): δ 174.45, 171.38, 148.16, 144.07, 141.0, 131.01, 130.98, 127.98, 127.72, 127.64, 127.44, 126.69, 126.65, 118.46, 115.05, 60.09, 58.38, 54.28, 42.33, 35.85, 20.14, 16.72. MS (ESI) m/z : 964.08, calculated for $[\text{M}]^+$: 964.14.

Synthesis of ERM-2

Compound **10** (100 mg, 0.18 mmol) was dissolved in dimethyl formamide (10 mL), followed by addition of compound **8** (81.80 mg, 0.18 mmol), ethylcarbodiimide hydrochloride (70.30 mg, 0.37 mmol) and *N*-methylmorpholine *N*-oxide (20 μ L). The reaction mixture was stirred overnight under room temperature. After confirmation by TCL, organic materials were extracted thrice with ethyl acetate. The combined extracts were washed thrice with water and once with brine, and dried over MgSO_4 . The crude mixture was then added with 1 ml of dichloromethane, 300 μ L trifluoroacetic acid (300 μ L), and anisole (20 μ L) was added into the reaction mixture, which was stirred under room temperature for 1 h. The solvents were removed under reduced pressure and subsequently crude mixture was purified by reverse phase HPLC to afford white solid **ERM-2** (21.10 mg, 14%). ^1H NMR (300 MHz, Acetone- D_6) δ (ppm): 7.12 (d, $J = 3.00$ Hz, 2H), 6.96-7.11 (m, 20H), 6.99 (d, $J = 8.37$, 2H), 6.64 (d, $J = 9.00$ Hz, 2H), 5.73 (t, $J = 4.83$ Hz, 1H), 5.05 (dd, $J = 3.00$ Hz, 6.00 Hz, 1H), 4.36 (s, 2H), 4.34 (d, $J = 7.95$, 1H), 4.13 (d, $J = 13.38$ Hz, 1H), 3.92 (d, $J = 13.38$ Hz, 1H), 3.88 (d, $J = 9.33$ Hz, 1H), 3.70-3.79 (m, 2H), 3.63 (q, $J = 16.38$, 2H), 2.63 (t, $J = 6.00$ Hz, 2H), 2.52 (t, $J = 6.00$ Hz, 2H), 2.01 (s, 3H). ^{13}C NMR (75 MHz, Acetone- D_6) δ (ppm): 173.22, 171.04, 169.58, 143.86, 142.32, 140.78, 137.71, 135.47, 133.94, 130.99, 127.72, 127.64, 127.62, 126.58, 126.44, 126.40, 126.37, 114.87, 59.52, 57.87, 53.98, 42.21, 38.81, 37.64, 34.23, 21.25. MS (ESI) m/z : 823.02, calculated for $[\text{M}]^+$: 823.00.

Enzyme Hydrolysis Assay:

Reaction mixtures (100 μ L each) containing ERM-1 and ERM-2 (10 μ M) were incubated separately with 50 nM of AmpC and TEM-1 respectively in 0.1 M PBS buffer (pH 7.4) at 37°C for 1 hr and subjected to fluorescent measurement. In control experiments, ERM-1 and ERM-2 was incubated with PBS buffer at 37°C for 1 hr. To study the enzyme specificity, the enzyme, TEM-1 and AmpC is pretreated with inhibitors CA and AZT (100 μ M) respectively and with ERM-1 and ERM-2 for fluorescent studies.

Enzyme Kinetics

The kinetic experiment was carried out at 37 °C in 0.1 M PBS buffer with pH 7.4. The fluorescence was measured using a fluorescence spectrometer. To a series of difference concentration of ERM-1 and ERM-2 (10 μ M to 50 μ M) were incubated separately with TEM-1 and AmpC (50 nM) and the fluorescence was monitored over a period of 60 minutes with excitation wavelength at 312 nm and emission wavelength at 478 nm. The values of the kinetic parameters (K_M and K_{cat}) were determined by least-squares fit of a double-reciprocal plot of the hydrolysis rate versus probe concentrations.

HPLC Analysis

ERM-1 and ERM-2 were incubated separately with TEM-1 and AmpC in PBS (pH 7.4, 0.1 M) at 37°C for 1 hr and subjected to HPLC analysis. The Bla (TEM-1 and AmpC 50 nM) hydrolysis of BSLP and NSPC were confirmed with analytical reverse-phase high performance liquid chromatography. RP-HPLC was performed on Alltima C-18 column (250 $\times$ 3.0 mm) at a flow rate of 1.0 mL/min. An eluting system consisting of A (water with 0.1% TFA) and B (acetonitrile with 0.1% TFA) was used under a linear gradient to elute the products, which was monitored by UV-Visible absorbance at 312 nm. The linear gradient

started from 80% solution A and 20 % solution B, changed to 20 % solution A and 80 % solution B in 40 minute and to 0 % solution A and 100% solution B in the following 5 minutes, and then back to 80 % solution A and 20 % solution B in the next 5 minutes.

Dynamic Light Scattering for size Distribution

The sizes and size population distributions of ERM-1 and ERM-2 after treatment with TEM-1 and AmpC (50 nM) respectively were determined on a Brookhaven 90 Plus Nanoparticle Size Analyzer. Dust-free solution vials were used for the aqueous solutions, and measurements were performed at an angle of 90° in room temperature.

Confocal Microscopic Bacteria Imaging

The overnight culture of bacteria suspension were diluted to 10^8 cells/ml and incubated with ERM-1 and ERM-2 (20 μ M). After washing the PBS, the bacteria cells were spotted on poly-L-lysine pretreated glass slides and immobilized with coverslips. Bacteria imaging tests were conducted with Zeiss LSM 800 confocal microscope. To study the enzyme specificity, the bacteria were pretreated with enzyme inhibitor CA and AZT and labeled with ERM-1 and ERM-2 for imaging studies.¹

Biofilm Confocal Microscopic Imaging

The bacteria cells were grown overnight in LB medium at 37 °C and subsequently diluted with LB in 1:100. Sterile glass cover slides were covered with 10 ml of bacteria suspension and incubated in 37 °C 24h. Bacteria suspension was discarded and slides were washed gently with 1 ml LB. ERM-1 and ERM-2 probes (10 μ M) were dissolved in LB medium and added to the slides for incubation for 1 hr 37°C. Slides were washed gently with sterile PBS (pH = 7.4, 0.1M) two times and observed by LSM 800 Zeiss Confocal Laser Microscope.

Flow-Cytometric Analysis

The overnight cultures of *E. coli* DH5 α , BL-21 and *E. cloacae* cells (10^8 CFU/ml) were first treated with 10 μ M of ERM-1 and ERM-2 for 1 hr and washed with PBS. The fluorescence of the cells was detected by a high sensitivity flow cytometer with a solid-state 355 nm continuous wave laser as excitation source. The emitted light was collected and split into two light paths for side scatter and blue fluorescence detection respectively.

ERM-1 Stability Test

The prepared ERM-1 (100 μ M) was incubated in several biological samples: 0.10 mL of PBS buffer (pH = 6.0, 7.4 and 8.0), LB (Lysogeny Broth) and 10% human serum for different time intervals at room temperature. At different time point, the mixture was re-suspended in MeOH (0.3 mL) and then centrifuged at 16,000 *g* for 5 min. The supernatant was analyzed by HPLC to evaluate the stability. There was no obvious ERM-1 degradation observed in PBS (in different pH), LB and 10% human serum (for 24 hours) indicated the sufficient stability of ERM-1 *in vitro* and living bacteria experiments.

2.3 Results and Discussions

As shown in scheme 1, it demonstrates the rational synthesis and design of the unique enzyme responsive molecule 1 (ERM-1). In general, the cephalosporin molecule is conjugated with the 4-aminothiophenol linker on its 3' position and further ligated with the TPE fluorophore. In order to achieve AmpC selectivity to be achieved, we further covalently linked a bulky methoxyimino group onto the 7' position of the cephalosporin scaffold³⁸. The presence of such a bulky group has been reported to slow the rate of hydrolysis of Class A Bla drastically owing to its smaller binding pocket. On the other hand, AmpC owing to its larger binding pocket that is able to fit in the bulkier ERM-1 moiety. To further prove the selectivity

is caused as a result of steric hindrance, we constructed another control molecule, ERM-2, which consist of a smaller acetyl functional group on the 7' position of the β -lactam ring structure. After the successful construction and synthesis of ERM-1 and ERM-2, the products were then purified using reverse phase high performance liquid chromatography (RP-HPLC) and subsequently characterized by nuclear magnetic resonance (NMR) and mass spectrometry (ESI-MS, ESI⁺). Such well-designed probes will then be further tested using TEM-1 and AmpC enzymes to examine its activities and systematic biological effect in bacteria. The probe characteristic and activities towards the 2 classes of enzymes were first examined by incubating ERM-1 and ERM-2 using 0.1 M phosphate buffer saline (PBS) solution at pH = 7.4 in the absence of Bla enzyme at 37 degrees for 1 h.

Fig. 2.1 (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).

After the separate treatment of the probes with TEM-1 and AmpC Bla respectively, a strong fluorescence change is detected at 478 nm wavelength (Figure 2.1). As observed, for ERM-1 upon the treatment process, there is an enhancement of approximately 120 folds' enhancement in fluorescence in the presence of AmpC but its counter part only observed ~40 folds enhancement TEM-1 incubated buffer solution. The results obtained have successfully demonstrated that enzyme hydrolysis has taken place and is capable of breaking the 3' position

aminothiophenol, which releases the TPE fluorophore⁴¹⁻⁴⁷. Due to the poor solubility of the TPE linkers, aggregation of the fragments take place leading to an enhancement of the fluorescence³²⁻³⁸. The increase in intensity of the fluorescence is mostly due to the restriction of the intramolecular rotation of the TPE linker fragments after the Bla hydrolysis.

Fig. 2.2. Fluorescence enhancement of (A) ERM-1 and (B) ERM-2 (10 μ M) in 0.1 M of PBS buffer, pH 7.4 incubated with TEM-1, AmpC (50 nM) and inhibitors Calvanic Acid (CA) and Aztreonam AZT (100 mM). (C) Normalized fluorescent intensity of ERM-1 and ERM-2 (10 μ M) with TEM-1 and AmpC (50 nM) (D) ERM-1 (10 μ M) in a range of [AmpC] 0~75 nM.

A similar treatment process is carried out for the probes in the presence of the enzyme inhibitor: Calvanic acid (CA) for TEM-1 and Aztreonam (AZT) for AmpC. The result obtained in Figure 2.2 clearly illustrated that AZT¹⁵ is able to greatly suppress the AmpC activity by the lower fluorescence intensity observed. This obtained data also clearly indicated that the probes are selective towards Bla enzyme hydrolysis and not by other enzymatic interactions. Furthermore, the normalized fluorescent intensity of ERM-2 with TEM-1 showed a significant higher fold increment (~2.5 times) when compared with ERM-1 incubated with TEM-1. Similar fold enhancement was observed for ERM-1 and ERM-2 with AmpC. These results unequivocally confirmed that ERM-1 is more readily cleaved by AmpC. Moreover, ERM-1 (10 μ M) was analyzed with a range of AmpC enzyme concentration (0-75nM) to further validate the fluorescence enhancement was a result of AmpC enzyme. The fluorescence saturates at 50 nM of AmpC which unequivocally confirms that the 50 nM AmpC is a suitable concentration fluorescent detection in ERM-1.

In addition, we performed another control experiment by incubating ERM-2 with Bla enzymes, AmpC and TEM-1. During this treatment process, we observed that there is no significant difference in fluorescence intensity change (~ 120 folds), in Figure 2.1. The results obtained successfully demonstrated that the presence of a bulkier functional group such as the methoxyimino group on the 7'position of cephalosporin is capable to differentiate and recognize AmpC Bla, leading to a higher fluorescence intensity. Furthermore, we carried out an enzyme kinetics analysis towards our probes ERM-1 and ERM-2 with varied concentrations between 10 – 100 μ M at 37 degrees in 0.1M PBS buffer solutions over a period of 1h (Figure 2.3).

Fig. 2.3. Enzyme kinetics of ERM-1 and ERM-2 with AmpC and TEM-1 (50 nM).

(A) ERM-1 and ERM-2 with AmpC. (B) ERM-1 and ERM-2 with TEM-1.

The obtained data revealed that ERM-1 could be enzymatically hydrolyzed by TEM-1 and AmpC with fairly reasonable Michaelis constant³¹⁻⁴⁰ ($K_m = 14.1 \mu\text{M}$ and $11.8 \mu\text{M}$) and catalytic constant ($K_{cat} = 3.01 \text{ min}^{-1}$ and 14.2 min^{-1}). In order to measure the selectivity of ERM-1, we further conducted another similar enzyme kinetic studies with our control probe, ERM-2. The enzyme kinetics for TEM-1 and AmpC were found to have $K_m = 11.4 \mu\text{M}$ and $10.3 \mu\text{M}$ and $K_{cat} = 15.9 \text{ min}^{-1}$ and 16.8 min^{-1} . These obtained results clearly suggested the probe, ERM-1, is capable to demonstrate selective recognition towards AmpC enzymatic activity.

The release of the TPE liners as a result of enzymatic interactions was further confirmed through HPLC analysis. As shown in figure 2.4, after treatment of ERM-1 with AmpC enzyme for 1 h, the molecule has been completely hydrolyzed to form the TPE linker with retention time at approximately 35 minutes. However, in the presence of TEM-1 Bla, majority of ERM-1 probe remained not hydrolyzed as indicated in the figure and with minimum TPE linkers observed. Furthermore, ERM-2 was completely hydrolyzed in the presence of AmpC and TEM-1, indicating that ERM-1 is more resistant to the hydrolysis of

TEM-1.

Fig. 2.4. HPLC analysis of ERM-1 and ERM-2 (100 μ M in 0.1M PBS) with AmpC and TEM-1 (50 nM) at absorbance 312 nm.

Moreover, to validate the aggregation of the TPE linkers took place after Bla activities, we performed a dynamic light scattering (DLS) experiment to monitor and checked the aggregated size of the fluorophores. The DLS measurements indicated the diameter size of the aggregated TPE linkers reached a size approximately 200 nm after AmpC treatment for ERM-1. However, a much smaller aggregation of $\sim$ 100 nm was observed for TEM-1 Bla hydrolysis reactions with ERM-1. Similar studies were carried out for ERM-2 with AmpC and TEM-1 Bla, the results obtained showed that the aggregated hydrodynamic size reaches approximately 200 nm which is similar to the complete hydrolysis as ERM-1 with AmpC. These obtained data further validated selective reaction of ERM-1 towards AmpC Bla enzyme and demonstrated the consistency of the results observed for the *in vitro* fluorescence studies (Fig 2.5).

Fig. 2.5. Hydrodynamic diameter of the residue of ERM-1 and ERM-2 (10 μ M in 0.1 M PBS) after interaction with AmpC and TEM-1 (50 nM) in DMSO/PBS (v/v=1/199). (A) ERM-1 with AmpC. (B) ERM-1 with TEM-1. (C) ERM-2 with AmpC. (D) ERM-2 with TEM-1.

Delighted by the results obtained, we decided to investigate the stability of the probe before testing the activity of the probe in live bacteria. Typically, we incubated the probe, ERM-1, in incubated with 10% of human serum and 10% of PBS solution for 24 hrs in a range of pH (6.0-8.0), so as to simulate the micro environment of the bacteria. The data revealed that the ERM-1 is very stable in this medium and do not demonstrates any form of decomposition in this environment (Figure 2.6).

Figure 2.6. HPLC analysis of ERM-1 (100 μ M) with PBS (pH = 6.0, 7.4 and 8.0), LB and Human Serum at absorbance 312 nm for 24 hrs.

Inspired by the positive data that we have obtained for both enzyme activity and stability test, we further investigated the applicability of the probe, ERM-1 and ERM-2 for live cells imaging. In this particular study, we used 2 different types of Gram- negative antibiotics resistant pathogen strains, *E. coli* BL-21 and *Enterobacter cloacae* (*E. cloacae*), owing to its high expression levels of TEM-1 and AmpC respectively. Furthermore, we have chosen a negative control, *E.coli* DH5 α strain (ATCC 53868) as it does not secrete any Bla for the purpose of hydrolyzing β -lactam ring, making the bacteria antibiotics susceptible. All the strains as described are then encoded for GFP plasmids which can be used to serve as a standard for bioimaging the distribution as well as the intrinsic behavior of these bacteria. Generally, 20 μ M of ERM-1 are separately added to these 3 strains of gram-negative bacteria and observed under confocal microscopy. After the incubation, a strong fluorescence emission was observed for ERM-1 with *E. cloacae*, which has high expression levels of AmpC. On the other hand, only weak fluorescence intensity was observed for TEM-1 high expression *E.coli* BL-21 strain (Figure 2.7)³⁸. More significantly, in our

control experiment with *E.coli* BL21 (TEM-1) and *E. cloacae* (AmpC) revealed a significant difference in fluorescent emission after the treatment with ERM-1. Pre-treating *E.coli* BL21 with its inhibitor CA and *E. cloacae* with AZT, virtually all fluorescent diminished (Fig 2.8). This result demonstrated that the ERM-2 is more sensitive to AmpC. expressed bacteria .

Fig. 2.7 (A) Confocal imaging of GFP expressed penicillin resistant *E. cloacae* and GFP expressed *E. coli* BL-21 bacteria, and antibiotic susceptible GFP expressed *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. ERM-1: Ex = 350/50 nm; Em = 450/50 nm. GFP bacteria: Ex =

400/450 nm; Em = 500/550 nm. Scale bar: 5 μ m.

Furthermore, we carried out a similar study using ERM-2 and the data revealed that there was no difference in fluorescence emission for both *E. cloacae* and BL-21 (Figure 2.9). These results that we have obtained unequivocally demonstrated that ERM-1 probe is capable to monitor and image AmpC Bla activity and label penicillin's resistant bacteria pathogens.

Fig. 2.8. Confocal imaging of penicillin resistant bacteria *E. cloacae* and *E. coli* BL21 pretreated with inhibitors (CA for *E. coli* BL-21 and AZT for *E. cloacae*) (100 μ M) 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.

Fig. 2.9. Confocal imaging of penicillin resistant bacteria *E. cloacae* and *E. coli* BL21, and antibiotic susceptible *E. coli* DH5 α bacteria with 20 μ M of ERM-2 in 0.1 M PBS, pH 7.4. Ex = 350/50 nm; Em = 450/50 nm. Scale bar: 5 μ m.

In order to quantify the data for the labeled AmpC expressed bacteria caused by AIE, these pathogens were subjected to flow cytometer (FCM). The 3 different bacteria: *E. cloacae*, *E. coli* BL-21 and *E. coli* DH5 α were first incubated separately with 10 μ M of ERM-1 and ERM-2 at 37 degrees for 1 h. The fluorescence signals were then collected at 478 nm. In figure 2.2 reveals a strong enhancement in fluorescence ($\sim$ 10 folds) after incubation of ERM-1 with AmpC expressed *E. cloacae*. However, a weaker fluorescence change was observed for the TEM-1 expressed *E. coli* BL-21 bacteria strains when compared against the negative control of *E. coli* DH5 α . Subsequently, we carried out a similar test for ERM-2 and the results revealed that there was no obvious difference in fluorescence between *E. coli* BL-21 and *E. cloacae* bacteria strains. These results clearly demonstrated that ERM-1 is capable to serve as a very reliable reporter probe for quantifying, labeling and measuring the intrinsic activity of AmpC express pathogen strains. Next, we investigated the feasibility of the probes, ERM-1 and 2 to monitor, label and localize on bacteria biofilms using different bacteria strains as models.

Typically, in our biofilm experiment we carried out the study using static biofilm. The bacteria strains were first cultured overnight in LB broth and diluted 100 times using the same medium and soaked with autoclave cover slits for 24 hours²⁰. During this process, the bacteria strains were first labeled with GFP plasmids to observe its formation and its distribution for the individual bacteria strains within the biofilms. The thin layer of biofilm formed on the surface of the cover slits were then treated separately with ERM-1 and 2 (20 μ M) for 1 h and then subjected to confocal microscopy imaging with emission wavelength at 478 nm and excitation wavelength at 350 nm. The figure shown in figure 2.10, showed the results for ERM-1 different staining patterns.

Fig. 2.10. Confocal imaging of penicillin resistant *E. cloacae*, *E. coli* BL-21, and antibiotic susceptible *E. coli* DH5α bacteria biofilms with 20 μ M of ERM-1 in 0.1M PBS, pH = 7.4. Ex = 350/50 nm; Em = 450/50 nm. Scale bar: 5 μ m.

As observed in the figure 2.10, there was significant fluorescence emission for class C Bla

AmpC expressed *E. cloacae* pathogens whereas only a very weak fluorescence signal was observed for biofilms formed by class A Bla TEM-1 expressed *E. coli* BL-21. In order to further confirmed the hydrolysis of ERM-1 is a result of AmpC Bla secreted by *E. cloacae*, we carried out an inhibitor experiment where the *E. cloacae* biofilm was first pre-incubated with AZT inhibitor and followed by treatment with ERM-1. As shown in figure 2.11, there was minimum fluorescence observed for the biofilm treated with the AZT inhibitor.

Fig. 2.11. Confocal imaging of penicillin resistant bacteria *E. cloacae* and *E. coli* BL21 biofilms pretreated with inhibitors (100 μ M) and 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.

Fig. 2.12. Confocal imaging of penicillin resistant bacteria *E. cloacae* and *E. coli* BL21, and antibiotic susceptible *E. coli* DH5α bacteria biofilms with 20 μM of ERM-2 in 0.1 M PBS, pH 7.4. Ex = 350/50 nm; Em = 450/50 nm. Scale bar: 5 μm.

More importantly, bacteria biofilms that have been incubated with ERM-1 revealed a higher fluorescent intensity in AmpC expressed pathogen biofilm as compared to TEM-1 expressed resistant bacteria biofilm. Furthermore, incubation with ERM-2 revealed similar fluorescence emission when observed under confocal microscope (Fig 2.12). All these obtained data clearly suggested that the rational design of the enzyme- responsive reporter molecule could accurately detect the specific target pathogens in the biofilm and for the controlled antibiotic resistance inactivation studies.

2.4. Conclusions

To sum up the work presented in chapter 2, we have successfully constructed a simple and practical approach for fluorescence bioimaging and localization of antibiotic resistant AmpC Bla producing pathogens strains in biofilms. The selectivity towards

AmpC Bla was achieved by installing a bulky functional group, methoxyimino group, on the 7' position of cephalosporins drug molecule¹³. This enzyme responsive reporter molecule could be used and served as a promising probe for the purpose of monitoring and bioimaging the AmpC activity for biofilm producing bacteria. More significantly, the localization of the ERM-1 in drug resistant pathogens could potentially help the medical communities for direct observation of pathogens in biofilm. At the same time, it can also be used to provide more effective treatment against biofilm related infections for both *in vitro* and *in vivo*.

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Chapter 3

Enzyme Triggered Near-Infrared Upconversion Nanoparticle for Beta-Lactamase imaging in Living Cells

3.1 Introduction

The widespread of bacterial infections in the human community has called for an urgent need to develop novel techniques that can provide accurate diagnostics and effective treatment of different types of pathogens. The wide spread of these bacteria infection diseases is commonly associated with extended-spectrum- β -lactamase (ESBL) which are increasing in clinical and veterinary medicine and posing a threat to the future health care. Due to the widespread use of antibiotics such as penicillin in clinics, these bacteria begin to develop resistance towards such drugs by secreting beta-lactamase, which acts as a form of defense mechanism that hydrolyzed the antibiotics before it reaches the bacteria cell wall¹⁻⁶. Therefore, the effective strategies that can investigate the intrinsic properties of such resistant enzyme (Bla) and obtain a better understanding of their molecular mechanism in bacteria pathogens are ongoing challenges in recent decades. β -lactamase do not exist in eukaryotes but can be easily expressed in it without any noticeable toxicity. Hence it has become an attractive biosensor for monitoring various biological processes and interactions in mammalian cells such as promoter/regulatory elements activity, inducible protein interactions, and ribozyme splicing reactions. Until now, numerous approaches have been designed to detect and monitor Bla activities in mammalian cells or bacteria pathogens^{6,7}. Among various methods, the light-mediated strategies have shown great superiority in achieving on-demand diagnostics of pathogen-induced diseases *in vitro* and *in vivo*, owing to their non-invasiveness and spatiotemporal precision upon a specific wavelength light irradiation²⁹. Typically, most of the developed methods involve the use of fluorescence resonance energy transfer (FRET)¹⁴⁻¹⁶ and the use of aggregation induced emission³⁶. After enzyme hydrolysis

reaction, the change in fluorescence signal can provide essential signals readout for investigating enzyme activity or quantitatively imaging antibiotic-resistant pathogens in living animals.

However, the commonly used excitation wavelength in the treatment of infections heavily rely on short-wavelength light (e.g., ultraviolet (UV) and visible light), which has undesirable background, serious photo-damage and limited tissue penetration depth owing to the inherent absorption/scattering of some endogenous light absorbers including water, lipids, and protein molecules including hemoglobin and oxyhemoglobin in living subjects²⁹⁻³². In order to further solve these problems, new light sources and photo-responsive systems are highly demanded. Nowadays, one inspiring approach to deal with these challenges is to utilize the long-wavelength near- infrared (NIR) light in the range of 700~1000 nm, termed as “optical window”. Typically, NIR light irradiation displays minimum absorption in living subjects, achieving better tissue penetration depth (e.g., < 10 cm) than UV or visible light (e.g. < 1 cm), which is also expected to exhibit minimal photo-damage to biomolecules, cells, and tissues³³⁻³⁵. Fortunately, in recent years, some novel nanomaterials with a large extinction coefficient in the NIR region, have recently become promising candidates in the field of NIR light-mediated theranostics, which could harvest NIR photons and generate cytotoxic moieties (e.g., reactive oxygen species, heat, etc.) for effective detection of pathogen-induced diseases in deep tissues in living systems²⁹.

Recently, lanthanide-doped upconversion nanoparticles (UCNs) have attracted much attention for bioimaging, treatment of cancers and bacteria biofilm annihilation based on their outstanding chemical and optical properties, including great biocompatibility,

high resistance to photobleaching and narrow-bandwidth emission. More importantly, they can serve as a promising nanotransducer with excellent tissue penetration depth *in vivo* to convert NIR excitation into a broad range of emissions from UV, visible to NIR region through a multi-photon upconversion process. Furthermore, the low toxicity of UCNs has enabled the broad applications in many medical researches as it enabled many studies to be carried out in both *in vitro* and *in vivo* settings. The high photostability of UCNs enabled a longer readout signal in cells, which provide a valuable benefit to monitor these Bla activities over longer extended periods of time with increased spatial resolution and higher sensitivity. These unique properties make lanthanide-doped UCNs particularly serve as a promising vector for biological sensing, biomedical imaging, and

Fig 3.1. Illustration of light mediated theranostic different depth of penetration in human body system from UV to NIR ⁷.

Recently, Ong and co-workers has successfully developed UCNs for the purpose of monitoring and also the detection of these pathogenic bacteria (Fig 3.2). Generally, the design of the particle takes advantage of the unique property of NIR light instead of the conventional shorter wavelength light such as the UV light which is more damaging to cells for the purpose of excitation²¹. The UCNs is modified with its surface coated a layer of a bacteria-specific

antibodies so as to increase the affinity of the particle with the pathogens. In the presence of NIR light, the GFP-expressed resistant bacteria will be observed to be labeled with the antibody coated UCNs. In this way, the bacteria would be successfully labeled and as a first demonstration for the labeling of bacteria using UCNs. In addition, the use of UCNs has also been applied for the purpose of eradicating biofilms formed by bacteria.

Figure 3.2. Diagram showing the use of upconversion nanoparticles for the labeling of Gram-negative bacteria²¹

In 2015, Qu and co-workers has successfully constructed a UCNs based particles for the purpose of releasing the conjugated D-amino acids labeled on the surface of the upconversion nanoparticle that could potentially disrupt the formation of biofilm which has been shown to be approximately a 1000 times more resistant to antibiotics (Fig 3.3)¹¹. In this manner, after the disruption of the biofilm, the pathogens would be more susceptible to the use of antibiotics and more readily annihilated.

Figure 3.3. Schematic diagram of a NIR multifunctional UCNPs triggered disassembling and annihilation of biofilm¹¹

However, the use of 980 nm irradiated UCNs offer certain drawbacks, which include overheating of cells and weaker penetration depth when performing cells and *in vivo* studies. Recently, many researches have documented the use of 808 nm particles offer a deeper penetration depth with lower heating effect¹⁷, which could potentially be more benign when performing live cell and *in vivo* imaging. As shown in figure 3.4, the relatively higher heat absorption by 980 nm is approximately 20 times higher and increment in temperature due to long exposure of 980 nm lasers is approximately 2 folds higher than 808 nm irradiation. Therefore, in order to perform deeper cell penetration imaging with lower cell-heating effect, UCN based particles, which rely on 808 nm for excitation is more preferred in recent times²³.

Figure 3.4. Absorption spectrum and temperature change of ultrapure water under different irradiation of wavelength²³.

As such to overcome the challenges of heavy reliant on short-wavelength (e.g., ultraviolet (UV) and visible light), which has undesirable background, serious photo-damage and limited tissue penetration depth, we constructed a 808 nm excitable UCNs for direct imaging and detection of Bla activities.

3.2 Design and Methods

Scheme 3.1. Enzyme triggered UCNP based Bla sensitive nanoparticle

As shown in the scheme 3.1, it illustrates the rational synthesis as well as the fabrication of the UCN based Bla activatable particles. In order to conduct a real-time Bla imaging, we synthesized the enzyme responsive probe (ERP) as shown in figure 3.5a and 3.5b. We first conjugated 4-aminothiophenol onto 3' position of the cephalosporin molecule for the purpose of further covalent ligation of the fluorescence acceptor Dabcyl group. Next, the lysine amino acid is then further covalently linked on the 7' position of the cephalosporin and conjugated with an activatable fluorescence resonance energy transfer (FRET) donor fluorophore, fluorescein isothiocyanate (FITC), at its side chain. The well-designed molecular probe would be used to test its activity using common Bla enzymes such as TEM-1 and AmpC Bla to examine its activities and also the systematic understanding of the biological effect in bacteria. Typically, in the absence of Bla, upon excitation of the UCNs at 808 nm, the UCN will upconvert the lower energy NIR light to a higher energy visible light range with a shorter wavelength of about 490 nm. This light source is then absorbed by the FITC molecule conjugated on the 7' position of the cephalosporin, which is covalently linked on the surface of the UCNs. The wavelength of 520 nm emitted by FITC would then be quenched by the quencher Dabcyl due to FRET, therefore no fluorescence would be observed. However, in the presence of Bla, the cephalosporins will be hydrolyzed at its 4-membered beta lactam ring by the serine-70 functional group by penicillin binding protein (PBP), which leads to the expulsion of the quencher Dabcyl. In this way, the FRET is disrupted and fluorescence activity will be detected.

Figure 3.5a. Synthetic steps of the Enzyme Responsive Probe (ERP) before conjugation to UCNPs

Figure 3.5b. Synthetic steps of the Enzyme Responsive Probe (ERP) before conjugation to UCNPs

As the absorption spectrum for Dabcyl overlaps very well (450 nm ~ 550 nm) with the

emission spectrum of FITC (490 nm~520 nm), no fluorescence should be observed for FITC. After the successful construction and synthesis of probe, the product was then purified using reverse phase high performance liquid chromatography (RP-HPLC) and subsequently characterized by nuclear magnetic resonance (NMR) and mass spectrometry (ESI-MS, ESI⁺).

Synthesis of 2

4-Aminothiophenol (150 mg, 1.20 mmol) was dissolved into anhydrous DCM and chlorotriphenylmethane (279 mg, 1.00 mmol) was added in one portion. Next, 0.5 ml of trifluoroacetic acid and reaction mixture was allowed to stir for 3 hours. After concentrating the solution, the compound was allowed to purify using column chromatography with eluent of Ethyl Acetate (EA): Hexane = 1:4 to give molecular intermediate **2**, 268 mg (73.1%). ¹H NMR (CDCl₃, 300 MHz): δ 7.20- 7.39 (m, 15H), 6.90 (d, J = 8.01 Hz, 2H), 6.66 (d, J = 7.6 Hz, 2H)

Synthesis of 3

2 (268 mg, 0.80 mmol) and 4-dimethylaminoazobenzene 4'-carboxylic acid (DABCYL) (208 mg, 0.80 mmol) were first dissolved in anhydrous pyridine (10.0 mL). Subsequently, phosphoryl chloride (116 mg, 0.8mmol) was then added dropwise at -10°C over 10 minutes. The reaction mixture was allowed to stir at - 10°C for 2 hour. After warming the mixture to room temperature, the mixture was then extracted with ethyl acetate, 10% sodium bicarbonate, water and finally brine. After concentrating the solution under reduced pressure, the reaction mixture was send to purify using flash column chromatography using an eluent of EA: Hexane = 1:1 to give 50.2 mg (10.2%) of the intermediate compound, **3**. ¹H NMR (CDCl₃,

300 MHz): δ 7.21-7.40 (m, 24H), 7.01 (d, J = 8.5 Hz, 2H), 6.74 (d, J = 9.0 Hz, 2H), 3.09 (s, 6H).

Synthesis of 4:

3 (30.1 mg, 0.046 mmol) was dissolved into 1 ml followed by the addition of 0.5 ml of TFA and 10% of triisopropylsilane. The reaction was allowed to stir for 1 hour at room temperature. After the starting material has disappeared from the thin layer chromatography, the mixture was then concentrated and used for the next step synthesis without any further purification.

Synthesis of 6:

Boc-lysine-OH (2 g, 8.12 mmol) was first dissolved in 2:1 CHCl_3/DMF (6.0 mL) follow by the addition of 6.0ml of TEA and finally 4-methyltrityl chloride (4-MttCl) (4.76 g, 17.0 mmol). The reaction mixture was allowed to stir overnight. After monitoring the reaction has completed by thin layer chromatography, the reaction was then quenched using 10.0ml of methanol. After extraction with ethyl acetate, water and brine and finally dried over with anhydrous magnesium sulphate, the compound was sent to purify using flash column chromatography to obtain a white solid (1.01g, 51%) of **6**. ^1H NMR (CDCl_3 , 300 MHz): δ 7.16-7.50 (m, 14H), 2.36 (s, 3H), 2.10 (s, 1H), 1.71 (s, 1H), 1.61(s, 3H), 1.47 (s, 9H), 1.31 (t, J =7.00 Hz, 4H).

Synthesis of 7:

6 (701 mg, 1.12mmol) first dissolved in 1:1 ACN/dioxane (10.0 mL) and next 7-Amino-3chloromethyl 3-cephem-4-carboxylic acid diphenylmethyl ester, ALCH.HCL, (700mg, 1.12mmol) was then added into the reaction mixture. Subsequently, 0.2ml of TEA was added to obtain a clear solution. Next, 1-ethyl-3-(3-

dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) (400 mg, 2.00 mmol) and also hydroxybenzotriazole (HOBt) (390 mg, 2.88 mmol). The mixture of solution was allowed to stir overnight and extracted with ethyl acetate, water and brine. The dried reaction mixture was then purified using normal phase chromatography to afford **7** (780.1 mg, 52%). ^1H NMR (CDCl_3 , 300 MHz): δ 7.00 - 7.30 (m, 24H), 5.85 (q, $J=5.00$ Hz, 1H), 3.70 (d, $J=18.0$ Hz, 1H), 3.39 (d, $J=18.0$ Hz, 1H), 2.25 (s, 3H), 2.10 (t, $J=6.40$ Hz, 2H), 1.50 (s, 9H), 1.61 (broad s, 4H), 1.30 (m, 2H)

Synthesis of **8**:

To a solution of **4** (22.8 mg, 0.058 mmol) in 0.1 ml of DMF, **7** (54.5mg, 0.059 mmol) was added in one portion. Next, a catalytic amount of NaI was then added to the reaction mixture and finally 2,6-lutidine was added and allowed to stir over night. After the starting material has disappeared from the thin layer chromatography, the solution was then extracted with ethyl acetate, water and brine. After drying under reduced pressure, the mixture was then purified using flash column to afford 30.1 mg (40.1%).

m/z 1239.8 [M^+]

Synthesis of **9**:

8 (30.1 mg, 0.0280 mmol) was dissolved in anhydrous DCM and followed by the addition of 1% TFA. The mixture was allowed to stir for 4 hours at room temperature, and then it was concentrated and prepared to be used as **9** for the next step reaction without further purification. m/z 984.17 [M^+]

Synthesis of **10** and Enzyme Responsive Probe (ERP):

The crude product **9** was first dissolved in 1ml of DMF and followed by the addition of FITC (10.0 mg, 0.0215 mmol) in one single portion. Finally and 5 μ l of DIEA was added drop wise while the reaction mixture was stirring vigorously. After stirring for 2 hours, the mixture was vacuumed dried as **10** and used to the next step synthesis without further purifications. The crude product **10** was dissolved in 1 ml of DCM followed by addition of 20% of TFA in 10% of anisole. It was then stirred vigorously for 1hr. After the starting material spot has disappeared, the mixture was sent to purify using reverse phase HPLC to obtain a fine red powder solid of **ERP** (10.0 mg, 0.009 mmol). m/z 1106.01

Conjugation of β -lactamase-sensitive probe onto UCNPs surface:

Figure 3.6. The fabrication and synthesis of UCNPs Bla sensitive nanoparticle

After confirming the construction of the **ERP**, the Bla activatable probe would be subsequently be used to conjugate onto the newly fabricated UCNs coated with polyetheneimine. **ERP** was conjugated onto the surface of UCNPs as shown in Fig 3.6. In the coupling reaction EDC.HCl and N- hydroxysuccinimide (NHS) were employed as the primary reagent for linking the ERP onto the surface of UCNPs. In additions, In order to increase the overall solubility of the UCNPs, the surface of the particle is coated with a layer of PEG₄-NH₂.

Synthesis of UCNs

1.1) Synthesis of NaYF₄:Yb/Er/Nd (18/0.5/1%) core upconversion nanocrystals (UCNs): The UCNs were obtained following a reference published before¹. Typically, 2 mL methanol containing RE(CH₃CO₂)₃ (RE = Y, Yb, Er and Nd), 3 mL oleic acid and 7 mL 1-octadecene were added in a 50 mL three-neck flask. The ratio of Yb/Er/Nd is 18/0.5/1% and the total lanthanide amount is 0.4 mmol. The mixture was heated to 150 °C for 60 min before cooling down to room temperature. Subsequently, a methanol solution (6 mL) containing NH₄F (59.3 mg) and NaOH (40.0 mg) was added and stirred for 30 min at 50 °C. Then methanol was evaporated and the solution was kept at 290 °C for 1.5 h under nitrogen atmosphere. After cooling down to room temperature, the core particles were precipitated using ethanol and collected through centrifugation several times after ethanol washing. Finally, the core particles were re-dispersed in 4 mL hexane.

1.2) Synthesis of NaYF₄:Yb/Er/Nd (18/0.5/1%)@NaYF₄:Nd (20%) core-shell UCNs: Typically, 3 mL oleic acid and 7 mL 1-octadecene were added in a 50 mL three-neck flask, then 2 mL methanol containing 0.4 mmol RE(CH₃CO₂)₃ (RE = Y/Nd (80/20%)) was also added in the solution. The mixture was heated to 150 °C for 60 min before cooling down to room temperature. The as-synthesized NaYF₄:Yb/Er/Nd core particles in 4 mL hexane were added along with a methanol solution (6 mL) containing NH₄F (59.3 mg) and NaOH (40.0 mg). The resulting mixture was stirred for 30 min at 50 °C. Then methanol was evaporated and the solution was kept at 290 °C for 1.5 h under nitrogen atmosphere. After cooling down to room temperature, the core-shell particles were precipitated using ethanol and collected through centrifugation several times after ethanol washing. The absolute quantum yield of core-shell UCN was 0.22 % under excitation at 808 nm (80 W cm⁻²) per the standard process.

1.3) Synthesis of PAA@UCNs: 300 mg of polyacrylic acid (PAA Mw 1800) and 30 mL DEG were added to a three-neck flask. The mixture was heated to 110 °C to form a clear solution. Then a hexane solution containing 100 mg core-shell UCNs was added slowly and the mixture was maintained at 110 °C for 1 h under nitrogen protection. After heating at 240 °C for 1.5 h, the resultant solution was cooled down to room temperature and precipitated with ethanol. The obtained PAA@UCNs was recovered by centrifugation (18000 rpm for 10 min) and washed three times with ethanol/water (1:1 v/v).

1.4) Synthesis of PEI/PAA@UCNs: The PAA-capped UCNs were covalently encapsulated with branched PEI. Briefly, 30 mg of PAA@UCNs in 5 mL water were activated by EDC.HCl (50 mg) and NHS (5 mg) to form the active succinimidyl ester for 30 min. Then the mixture was added slowly to 10 mL polyethylenimine (PEI Mw 25000 branched) 2 mg mL⁻¹ in PBS (pH 7.4) with 30 min sonication. After 24 h magnetic stirring, the PEI/PAA@UCNs was purified by centrifugation (18000 rpm for 10 min) and washed three times with ethanol/water (1:1 v/v) to remove excess reactant.

1.5) Synthesis of UCNPs@ERP: The PEI/PAA@UCN were covalently conjugated to molecule 11 (enzyme responsive probe, ERP). 30mg of PEI/PAA@UCN is dissolved in ml of distilled water. Subsequently, ERP, EDC.HCl (50mg) and NHS (5mg) were added to the mixture. The reaction was allowed to stir overnight at room temperature pressure. The UCNPs@ERP was purified by by centrifugation (18000 rpm for 10 min) and washed three times with ethanol/water (1:1 v/v) to remove excess reactant.

Enzyme Hydrolysis Assay:

Reaction mixture (100 μ L each) containing ERP (10 μ M) and UCNPs@ERP (1.5mg/ml) were incubated separately with 50 nM of AmpC and TEM-1 respectively in 0.1 M PBS buffer (pH 7.4) at 37 °C for 1 hr and subjected to fluorescent measurement. In control experiments, ERP (10 μ M) and UCNPs@ERP (1.5mg/ml) were incubated with PBS buffer at 37 °C for 1 hr. To study the enzyme specificity, the enzyme, TEM-1 and AmpC is pretreated with inhibitors CA and AZT (100 μ M) respectively and with ERP (10 μ M) and UCNPs@ERP (1.5mg/ml) for fluorescent studies.

Confocal Microscopic Bacteria Imaging

The overnight culture of bacteria suspension were diluted to 10^8 cells/ml and incubated with ERP (10 μ M). After washing the PBS, the bacteria cells were spotted on poly-L-lysine pretreated glass slides and immobilized with coverslips. Bacteria imaging tests were conducted with Zeiss LSM 800 confocal microscope. To study the enzyme specificity, the bacteria were pretreated with enzyme inhibitor CA and AZT and labeled with ERP for imaging studies.¹

Confocal Microscopic Mammalian cells Imaging

Bla overexpressing Jurkat-CMV- β -lactamase and Bla deficient Jakart cells were seeded with a cell density of 1×10^5 in an ibidi confocal μ -dish (35 mm, plastic bottom) in 1 mL DMEM media which was incubated NIH/3T3 cells were seeded with a cell mL^{-1} for 24 h at 37 °C. The seeded cells were subsequently treated with UCNPs@ERP and incubated for 1 h. As controls, the HT-29 cells and Jakart cells were treated with UCNPs@ERP in

the present of Bla inhibitor. Mammalian cells imaging tests were conducted with Zeiss LSM 800 confocal microscope.

3.1 Results and Discussions

The enzyme responsive probe (ERP) was synthesized and purified using reverse HPLC to obtain a yield of approximately 5%. In order to test the feasibility of the UCNs based cephalosporin FRET molecule; we first perform a Bla hydrolysis on ERP without the conjugation of the UCNs. This is to ensure that the probe is activatable in present of Bla and not other non-specific interaction caused by its micro-environment.

We have selected TEM-1 and AmpC Bla owing to its high expression levels in many hospital infectious diseases, which include bacteremia, urinary tract infections as well as nosocomial pneumonia. To validate the activity of ERP towards Bla, ERP was first dissolved in 10% PBS buffer at pH = 7.0 and subsequently incubated with 50 nM of TEM-1 and AmpC at 37 °C for 1hr. As shown in figure 3.7, in the absence of Bla, the fluorescence activity of the probe is virtually absent. This is due to the efficient FRET effects, which caused the fluorescence, emitted by the FITC fluorophore to be effectively quenched by the Dabcyl quencher. However, in the presence of Bla, the fluorescence signal was observed to increase gradually and reaching its maximum value at the mark of 90 minutes. The fluorescent intensity of the fluorescent did not increase any further between 90 to 120 minutes. The total fold enhancement as compared to the absence of Bla was about 150 folds for both TEM-1 and AmpC Bla. These results demonstrated that the enzyme hydrolysis could break the linker at the 3' position of cephalosporin, thus result in the effective release of the quencher molecule. Then subsequent fluorescence enhancement at 520 nm.

Figure 3.7. Fluorescent emission of enzyme responsive probe (ERP, 10 μ M) before and after incubation with TEM-1 and AmpC Blas in PBS (pH = 7.4).) with a) TEM-1 and b) AmpC. Excitation wavelength 420nm.

However, in order to prove ERP is sensitive towards Bla but not other non-specific interactions, we conducted an inhibitor test by first preincubating CA (Class A Bla inhibitor) with TEM-1 and AZT (Class C Bla inhibitor) with AmpC. As observed in figure 3.8, little to no fluorescence was observed in the presence of Bla enzymes. The obtained result clearly indicated that ERP is sensitive only to the enzyme activity by Bla but not other forms of non-specific hydrolysis interactions. Following the confirmation of the sensitivity of ERP towards Bla enzyme, we further carried out the conjugation of the probe to the surface of UCNs for the purpose of imaging Bla activity in buffer assays.

Figure 3.8. Enzyme responsive molecule (ERP) fluorescent emission in the present and absent of inhibitor for TEM-1 and AmpC

After the successful conjugation of the lanthanides-doped UCNs with ERP, the labeling efficiency is found to be at about 2.0% (Figure 3.9). Subsequently, the fluorescence activity with Bla was carried out and examined. Typically, the 1.5 mg/ml of UCNs particles was dissolved in 10% PBS buffer solution and 50 nM of TEM-1 was added into the mixture and the fluorescence activity was observed over a period of 120 minutes. As shown in figure 3.10, the fluorescence intensity shows a increment of approximately 1 fold at 520 nm after incubation with TEM-1. Upon longer incubation period, no further fluorescence enhancement was observed and this observation was consistent to the buffer assay result obtained between ERP and Bla hydrolysis.

Figure 3.9 Loading efficiency of UCN@ERP. Fluorescent intensity of UCN@ERP at different concentration (2.5 ~ 75 μM)

a)

b)

Figure 3.10. Upconverted fluorescent emission of conjugated enzyme responsive probe for a) TEM-1 b) AmpC

In order to validate that the result obtained is the hydrolysis result and not by other non-specific interaction, we further carried out an inhibitor experiment where TEM-1 is first pretreated with CA, followed by the addition of the UCNs based ERP. As shown in the figure 3.11, the fluorescence enhancement at 520 nm has been greatly suppressed. Moreover, another similar study was carried out using AmpC Bla, the result obtained is consistent with the ones obtained as observed in TEM-1 hydrolysis reaction with the UCNs based ERP.

Figure 3.11. Upconverted fluorescent emission of conjugated enzyme responsive molecule (UCNPs@ERP)

Delighted by the data obtained and to test the viability of the probe to be used in cells study, we first examined the fluorescence activity of the probe, ERP, in Bla expressed bacteria. In generally, two separate groups of bacteria were chosen for this study: antibiotic-resistant bacteria and antibiotics non-resistant bacteria. The bacteria types are made of 3 different types of gram-negative bacteria: a control antibiotics

susceptible *E. coli* DH5 α and two resistant pathogens strains which consist of high expression of TEM-1 Bla, *E.coli* BL-21, and high expression of AmpC, *Enterobacter cloacae* (*Enter. Cloacae*). These 3 different strains of bacteria are chosen for this study is due to its wide spread in human community and also its numerous infection diseases such as Urinary tract infections and nosocomial pneumonia in clinics.

Figure 3.12. Fluorescent imaging of molecular responsive probe with antibiotic resistant bacteria

Generally, the study is carried out by first preparing 10^8 of bacteria cells ($OD_{0.5}$) for each type of bacteria strains and incubate 10 μ M of ERP for about 1 h in 10% PBS solution

at 37 °C. Next, the incubated bacteria were washed twice with PBS buffer and subjected with confocal microscopy for fluorescence imaging. As shown in figure 3.12, the control bacteria DH5 α shows virtually no fluorescence activity in the presence of the probe. However, strong fluorescence activity was observed in *E.coli* BL-21 and *Enter. Cloacae*. The results clearly demonstrated that the probe ERP is capable of monitoring the activity of the resistant enzyme Bla.

Delighted by the result, we examine the feasibility of the UCNs@ERP capability to monitor the activity of Bla in living cells by carrying out the study into mammalian cells. Generally, we have chosen 2 different types of cells lines: a negative control Jurkat cells which does not have any expression of Bla enzymes while the other mammalian cells (Jurkat-CMV- β -lactamase) with high TEM-1 Bla expression. The mammalian cells are cultured to a density of 10^6 for both Jurkat cells with no Bla expression and Jurkat-CMV- β -lactamase and were then incubated with 50 μ g/ml of the UCNs@ERP for 1 h and followed by confocal microscopy imaging.

As observed in figure 3.12, Jurkat cells with no Bla expression has a minimum fluorescence observed while Jurkat-CMV- β -lactamase has a significant increase in fluorescence intensity. The enhancement in fluorescence intensity is likely due to the hydrolysis of the beta lactam ring by the serine residue that leads to the release of the Dabcyl, which disrupts the FRET between the donor and acceptor. At the same time, an inhibitor experiment was carried out in order to examine the hydrolysis of the probe is a result of the Bla activity and not other non-specific enzyme hydrolysis. In figure 3.13, there is minimum to no fluorescence observed for the mammalian cells, Jurkat Bla, pretreated with CA which is an inhibitor for TEM-1 Bla. This result obtained clearly indicated that UCNs@ERP is sensitive to the activity of Bla enzyme.

Figure 3.13. Fluorescent imaging of Jarkart with Bla (+) and Bla (-) expression.

Furthermore, to validate the increase in fluorescence activity was caused by Bla activity and not by other forms of unspecified proteins or enzyme interactions by the mammalian cells, we carried out a cell lysate experiment. Generally, mammalian cells Jarkart (+Bla) and Jarkart (-Bla), were lysate by a cycle of freeze and thaw methods¹⁶. The supernatant collected was then treated with UCNs@ERP probe. In Figure 3.14, a one fold enhancement of fluorescence was observed for the Jarkart mammalian cell lines with Bla expressions. Importantly, there was no obvious fluorescent enhancement detected in Jarkart (+Bla) cells pre-treated with CA and control Jarkart mammalian cells with no Bla expression. These results unequivocally indicated the intrinsic capability of the rationally developed UCNs@ERP to selectively report Bla activities in gene express cells without the interference by other non-specific interactions within the mammalian cells.

Figure 3.14. Cells lysate experiments treated with UCNPs conjugated enzyme responsive probe.

3.4. Conclusions

In conclusion, we have successfully developed and constructed an UCN-based molecular probe for monitoring the activities of gene-reporter cells expressing Bla enzyme at higher wavelength with lower photo-damage and better tissue penetration. The lower heat absorption of the UCNs at 808 nm has offered a tremendous advantage during the process of imaging as it eliminates the possible threats of overheating the cell lines and eradicating the problems associated with photo-toxicity²⁹. This work present the first-time demonstration of using UCNs for the purpose of monitoring and detecting these resistant bacteria in cells. The use of such strategies combined with other continuous development of antibiotic pipelines will allow medical doctor to achieve a more significant breakthrough and improvement of better antibiotics to battle against these resistant bacteria strains.

3.5 References

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Chapter 4

***In vivo* Metabolic Mediated Selective Covalent Localization Reporter for Pathogenic Bacteria Recognition**

4.1 Introduction

Currently, the emergence of antibiotics resistant bacteria, particularly towards broad-spectrum β -lactam antibiotics, has been a major public health concern due to the misuse and overuse of antibiotics. One of the main causes of such rapid increase in resistance is the high-level expression of β -lactamase (Bla), a family of bacteria enzymes produced as a means of self-defense that inactivates β -lactam antibiotics, rendering these bacteria ineffective towards conventional therapy. Among the different classes of Bla, Class A and C variants are the most commonly encountered¹⁻⁷. Despite the leap forward in biomedical research, critical challenges remain towards the design of targeted small molecules for specific recognition of the far less investigated Class C Bla, which poses significant clinical threats, in comparison to the well-researched Class A Bla. Established clinical methods to detect Bla include the use of susceptibility tests such as Modified Hodge Test (MHT) and double-disk synergy test (DDST)^{8,9}. However, these methods are often time-consuming and lack the selectivity for accurate diagnosis of bacterial infections. Therefore, more efficient and specific targeted approaches are desirable aspects in the quest of developing novel concepts for detecting these resistant bacteria. The use of fluorescent imaging via small molecules probes and fluorescent proteins represents an established method for detecting/monitoring of resistant bacteria *in vitro* and *in vivo* studies.

Several fluorogenic probes for Bla have been reported¹⁴⁻¹⁸ and discussed in chapter 2 and 3 through the use of AIE molecule and NIR imaging of mammalian cells expressing Bla. However, very few works have been done in selective localization of small fluorescent probes

using infrared or near-infrared fluorescence imaging. Using infrared fluorophores for localization of small molecules in resistant bacteria offer numerous advantages as it has less photo damage to cells, produces lower auto fluorescence background noises and higher sensitivity. More importantly, it offers deeper cell penetration and less light scattering than visible light which makes it a better candidate for living animal imaging.

One of a useful technique for direct localization of probes is the use of bio-orthogonal chemistry in the concept of metabolic labeling. It has been proven to be an effective strategy and has been widely used in biomedical applications. In general, metabolic mediated labeling enables the covalent ligation of small molecule probes onto cellular structures for the direct localization and selective bio-imaging of the dynamic intrinsic microenvironment of cell and bacteria. Most existing small molecules that are available for direct imaging of Blas studies often lacks the capacity for the selective localization in resistant bacteria strains. Moreover, the conventional methods for cell surface labeling involves the conjugation of fluorescent proteins (>130kDa) to bacteria which may potentially perturb the endogenous properties of the proteins. Therefore, the need for novel fluorophores that can perform the task is greatly desired¹⁸. As such to overcome these challenges, metabolic mediating labeling represents a great alternative towards the directed localization onto the resistant bacteria surface for imaging Class C Bla activities

In this work, a metabolic mediated bio-orthogonal probe sensitive towards AmpC Bla enzyme for the sensing of resistant bacteria has been designed and prepared to perform *in vivo* covalent localization of cephalosporin's at the infected region. Different from the process which involves non-specific Bla targeting, such a unique construct can respond to AmpC enzyme and undergo covalent bond formation with the metabolized cellular structure, overcoming

problems associated with random diffusion and serving as a robust fluorogenic probe to image these bacteria pathogens in real time. More significantly, compared to the non-metabolic mediated process, the enzyme triggered ligation process will enable a more robust fluorescent read out signal. Such enhancement enables a more dynamic visualization of AmpC Bla expressed resistant bacteria *in vivo* and *in vitro* for clinical studies²¹⁻²³.

4.1 Design and Methods

Scheme 4.1. Illustration of the metabolic mediated bioorthogonal covalent localization of Enzyme in infected areas. Upon the incorporation of the D-Ala-Azide in the resistant bacteria, Enzyme Probe 1 (EP-1) conjugated with DBCO will undergo a copper-free click reaction on the surface of the N_3 -tagged bacteria. After AmpC Bla activation by the pathogenic bacteria, the dark quencher BHQ-3 would be expelled from the cephalosporin leading to an enhancement of fluorescence for specific detection.

In scheme 4.1, it represents the rational design of our beta-lactamase sensitive probe with bio-orthogonal labeling capabilities for selective labeling of bacterial cell peptidoglycan modified with azide functional groups via 3-azido-d-alanyl metabolic incorporation. The probe consists of a Dibenzocyclooctyne moiety containing modified cephalosporin backbone flanked by an activatable Forster Resonance energy transfer (FRET) pair consisting of a NIR

activated fluorophore Cy5.5 and a NIR dark quencher BHQ-3. Cy5.5 was chosen for usage as a fluorophore, given its NIR excitability for increased penetration of excitation wavelengths aiding effective usage of the probe towards *in vivo* applications. Pre-quenching of the Cy5.5 fluorophore was achieved through the use of a dark quencher BHQ-3 moiety to increase signal to noise ratio of the probe. As such, labile *p*-aminothiophenol linker bearing the BHQ-3 moiety was introduced to the 3' position of the beta-lactamase sensitive cephalosporin backbone. A lysine linker was conjugated onto the 7' amino group of the beta-lactam ring for the subsequent installation of a Cy5.5 fluorophore and Dibenzocyclooctyne (DBCO) functional group, which would allow for effective click coupling of the probe onto N₃-tagged cell peptidoglycan. Activation of the probe upon successful β -lactamase hydrolysis is expected to result in spontaneous elimination of the BHQ-3 quencher moiety and subsequent visualization of strong fluorescence at $\lambda_{em}=710\text{nm}$ upon excitation. (Scheme 4.1).

Considering steric bulk on the 7' amino end of the cephalosporin backbone that can be recognized of cephalosporins by Class C Bla but not Class A Bla, we hypothesize that the presence of bulky substituents such as Dibenzocyclooctyne moiety conjugated onto the proximity of the 7' amino end of the beta-lactam ring would present a steric effect and thus result in a greater selectivity towards the sensing of Class C Bla producing bacteria. In comparison, a similar probe EP-2 was similarly synthesized with absent of DBCO moiety. Upon the successful synthesis and characterization of EP-1 and EP-2 via NMR and Mass Spectrometry (ESI-MS), the obtained probe was then subjected to further *in-vitro* and subsequent *in-vivo* investigations.

Figure 4.1. Synthesis of EP-1 and EP-2

Synthesis of compound 2:

Boc-lysine-OH (1.00 g, 4.06 mmol) and triethylamine (2.9 mL, 16.30 mmol) were added to a

solution of 4-methyltrityl chloride (2.38 g, 8.13 mmol) in 2:1 $\text{CHCl}_3/\text{N,N}$ -dimethylformamide solution (6.0 mL) and stirred under ambient conditions for 4 hours. Upon reaction completion monitored by TLC, methanol (5 mL) was then added to quench the reaction. The reaction mixture was then diluted with ethyl acetate (30 mL), washed with water (3 x 30 mL), brine (2 x 30 mL), the organic layers collected and dried with anhydrous magnesium sulfate before concentrated under reduced pressure. The residue obtained was purified by flash chromatography (EtOAc: Hexane = 1:1) to yield the product as a white crystalline solid. (1.21 g, 59%). ^1H NMR (CDCl_3 , 300 MHz): δ 7.12–7.47 (comp, 14H), 2.35 (s, 3H), 2.07 (s, 1H), 1.68 (br, 1H), 1.55 (br. s, 3H), 1.43 (s, 9H), 1.29 (t, $J=7.11$ Hz, 4H). calculated for $\text{C}_{31}\text{H}_{38}\text{N}_2\text{O}_4^+$: 502.28; found $^+$: 503.38

Synthesis of compound 3:

Compound **2** (363 mg, 0.722 mmol) was dissolved in a mixture of 1:1 $\text{CH}_3\text{CN}/1,4$ -Dioxane solution (24.0 mL) to obtain a colourless solution. Triethylamine (0.1 mL, 0.722 mmol), followed by 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid diphenyl-methyl ester under ambient conditions hydrochloride (7-ACMA) (326 mg, 0.722 mmol) was then added and stirred for 5 minutes. After which, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl) (208 mg, 1.08 mmol) and hydroxybenzotriazole (HOBt) (195 mg, 1.44 mmol) was added to the reaction mixture and stirred for 24 hours under ambient conditions. Upon reaction completion monitored through TLC, the reaction mixture was concentrated under reduced pressure,

the residue extracted with ethyl acetate (30 mL) and washed with water (30mL x3), brine (30mL x 2) and dried with anhydrous magnesium sulfate. The mixture was then concentrated under reduced pressure and the residue purified by flash chromatography (EtOAc: Hexane = 2:3) to yield the compound as a pale off- white solid. (0.319g, 47.2%)
 ^1H NMR (CDCl_3 , 300 MHz): δ 6.98 -7.32 (comp, 24H), 5.79 (q, $J=4.92$ Hz, 1H), 3.58 (d, $J=18.2$ Hz, 1H), 3.42 (d, $J=18.3$ Hz, 1H), 2.30 (s, 3H), 2.12 (t, $J=6.39$ Hz, 2H), 1.40 (s, 9H), 1.58 (br. s, 4H), 1.25 (comp, 2H) ^{13}C NMR (CDCl_3 , 300 MHz): ; HRMS (m/z) calculated for $\text{C}_{52}\text{H}_{55}\text{ClN}_4\text{O}_5\text{S}^+$: 882.36; found: 883.12.

Synthesis of compound 4:

Compound 3 (319 mg, 0.362 mmol) was dissolved in N,N dimethylformamide (1.0 mL) to obtain a pale yellow solution. To it, NaI (45.8 mg, 0.306 mmol) was added and the reaction was stirred under ambient conditions for 5 minutes. After which, 4-aminothiophenol (22.8 mg, 0.183 mmol) was added, followed by 2, 6-lutidine (19.1 μL , 0.164 mmol) into the reaction mixture. The reaction mixture was then stirred under ambient conditions for 12 hours. Upon reaction completion monitored by TLC, the reaction mixture was extracted with ethyl acetate (30mL), washed with water (3 x 30 mL), brine (2x 30mL) and dried with anhydrous magnesium sulfate. The mixture was then concentrated under reduced pressure and purified with flash chromatography (EtOAc: CH_2Cl_2 = 1:4) to yield the compound as pale brown residue (0.0122g ,34.7%). ^1H NMR (CDCl_3 , 300 MHz): δ 6.78 -7.24 (comp, 30H), 5.68 (q, $J=4.45$ Hz, 1H), 3.26-4.14 (comp, 6H), 2.60 (s, 2H), 2.30 (s, 2H), 2.09-2.14 (comp, 2H), 1.42-1.45

(comp, 21H) calculated for $C_{58}H_{61}N_5O_5S_2^+$: 971.41; found: 972.5

Synthesis of compound 5:

5

Compound **4** (122 mg, 0.125 mmol) was dissolved in N, N dimethylformamide (2 mL) to obtain a dark yellow solution. To it, Black Hole Quencher®-3 (BHQ®-3) (45 mg, 0.125 mmol), (1-[Bis (dimethylamino) methylene]-1H-1, 2, 3-triazolo [4, 5-b] pyridinium 3-oxidhexafluorophosphate) (HATU) (47.5 mg, 0.125 mmol) and N-Methylmorpholine N-oxide (NMO) (16.0 μ L, 0.125 mmol) were added and the reaction mixture stirred at 25°C for 24 hours. Upon reaction completion monitored by TLC, the reaction mixture was extracted with ethyl acetate, washed with water (3 x 30mL), brine (2 x 30mL) and dried with anhydrous magnesium sulfate. The mixture was then concentrated in vacuum and purified with column chromatography (EtOAc: Hexane = 1:1) to yield the compound as a dark blue residue (0.095g, 50.3%) 1H NMR ($CDCl_3$, 300 MHz): δ 7.97 – 7.58 (comp, 3H), 7.55(br. s, 1H) 7.15 – 7.41(comp, 39H), 7.08(d, J = 8.0Hz, 1H), 6.83(s, 1H), 6.64(d, J = 9.1Hz, 1H) 5.68(q, J = 5.1Hz, 1H), 4.94(dd, J = 24.1Hz, 4.7Hz, 1H), 2.81-3.47 (comp, 13H), 2.30(s, 3H) 2.16 (s, 1H), 1.79 (br. s, 4H), 1.43 (s, 9H), 1.25 (t, J = 7.1 Hz, 6H) calculated for $C_{91}H_{94}N_{11}O_7S_2^+$: 1516.68; found: 1517.88

Synthesis of compound 6: **6**

Compound **5** (54.5 mg, 0.0359 mmol) was added to a solution of 2% TFA in DCM (2mL) to obtain a deep blue solution. The solution was stirred for 2 hour at 0°C. Upon reaction completion monitored by TLC, the reaction mixture was concentrated under reduced pressure and dried under high vacuum to obtain compound **6** as a crude mixture and used for the subsequent reaction without any further purification. calculated for $C_{71}H_{78}N_{11}O_7S_2^+$: 1260.55; found: 1261.

Synthesis of compound 7: **7**

Compound **6** (45.3 mg, 0.0359 mmol) was dissolved in N, N Dimethylformamide to obtain a dark blue solution. Cy5.5-N-hydroxysuccinimide ester (Cy5.5 NHS ester) (5.66 mg, 0.00790 mmol) and N,N-Diisopropylethylamine (2.76 μ L, 0.0158 mmol) was added and the reaction mixture was stirred under ambient conditions for 4 hours. Upon reaction completion monitored by TLC, the mixture was then purified via Reverse Phase - HPLC and monitored by UV-Vis absorbance at $\lambda_{\text{abs}} = 650, 670$ nm and the collected eluent lyophilized to yield the product as a fine blue solid (2.73 mg, rho % over 2 steps).

Calculated for $\text{C}_{110}\text{H}_{121}\text{N}_{18}\text{O}_8\text{S}_2^{2+}$: 908.45; found: 909.55

Synthesis of EP-2:

Compound **7** (2.73 mg, 0.00149 mmol) was dissolved in 20% TFA and 5% anisole in CH_2Cl_2 and stirred under ambient conditions for 1 hour. Upon reaction completion monitored by TLC, the solvent was evaporated under reduced pressure and the mixture was then purified via Reverse Phase - HPLC and monitored by UV-Vis absorbance at $\lambda_{\text{abs}} = 650, 670$ nm and the collected eluent lyophilized to yield the product **EP2** as a fine blue solid. (3 mg, 21 %). calculated for $\text{C}_{92}\text{H}_{103}\text{N}_{13}\text{O}_6\text{S}_2^{2+}$: 774.88; found: 775.12

EP-1 (2.58 mg, 0.00149 mmol) was dissolved in N, N-dimethylformamide (1mL), to which Dibenzocyclooctyne-N-hydroxysuccinimidyl ester (2.06mg, 0.00512 mmol) and N, N-Diisopropylethylamine (0.447 μ L, 0.00267 mmol) to the reaction mixture. The reaction mixture was stirred under ambient conditions for 12 hours. Upon reaction completion monitored by TLC, the solution was then purified via HPLC and monitored by UV absorbance at 650 nm and 670 nm. After lyophilisation, the product was obtained as a fine blue solid (0.215 mg, 0.116 μ mol). (1.1 mg, 20 %). calculated for $C_{112}H_{114}N_{14}O_8S_2^{2+}$: 923.92; found 924.12.

Enzyme triggered probe activation of EP-1 and EP-2

The reaction scheme of enzyme triggered fluorescence activation of EP-1 and 2 was shown on Scheme 4.1. The stock solution of EP-1 and EP-2 in dimethyl sulfoxide was diluted with Phosphate buffer Saline (PBS) to obtain 10 μ M of respective probe solution in PBS. The emitted fluorescence in the quartz cuvette was recorded upon incubating the probe solution in PBS solution with TEM-1 Bla and AmpC Bla respectively (10nM) in 10% PBS and excitation of the contents of the cuvette at λ_{ex} = 670nm at 37°C over a time period of 1 hour. In the negative control experiment, The emitted fluorescence in

the quartz cuvette was recorded upon incubating MLP-1 in PBS solution with TEM-1 Bla with a Class A Beta-lactamase inhibitor Clavulanic Acid (100 μ M), as well as AmpC Bla with a Class C Beta-lactamase inhibitor Aztreonam respectively (100 μ M) in 10% PBS at 37°C over a time period of one hour under excitation of the contents of the cuvette at $\lambda_{\text{ex}} = 670\text{nm}$. In both experiments, both the excitation and emission slit widths were kept at 10.0 nm.

Bioimaging of bacteria labeled with DBCO-Cy3 via bio-orthogonal metabolic labeling

Bacteria cells were seeded and grown on Brain Heart Infusion broth in centrifuge tubes and shaken overnight, upon which OD₆₀₀ = 0.5 of bacteria cells was resuspended in Brain Heart Infusion (BHI) broth in an eppendorf tube. 3-Azido-D-Alanine hydrochloride (10mM) was added to the bacteria sample and subsequently incubated at 37°C for one hour to allow for the metabolic uptake of 3-Azido-D-Alanine onto bacteria peptidoglycan. After which, the medium was removed and the bacteria was washed with PBS (pH 7.4) thrice. Upon washing, the bacteria was re-suspended in HEPES Buffer (pH 7.4, 10mM EDTA) and incubated for 1 hour with DBCO Cy3, followed by washing with PBS thrice and re-suspension of the bacteria sample in HEPES buffer before plated onto poly L-lysine coated glass slides and visualized on a confocal microscope under excitation wavelength $\lambda_{\text{ex}} = 550\text{nm}$. The fluorescence imaging data of bacteria upon the two-step metabolic incorporation of azide and bio-orthogonal click coupling of DBCO-Cy3 were shown in Figure 4.3.

Bioimaging of resistant bacteria labeled with EP-1 via bio-orthogonal metabolic labeling

Bacteria cells were seeded and grown on Brain Heart Infusion broth in centrifuge tubes and shaken overnight, upon which $OD_{600} = 0.5$ of bacteria cells was re-suspended in Brain Heart Infusion (BHI) broth in an eppendorf tube. 3-Azido-D- Alanine hydrochloride (10 mM) was added to the bacteria sample and subsequently incubated at 37 °C for one hour to allow for the metabolic uptake of 3-Azido-D- Alanine onto bacteria peptidoglycan. After which, the medium was removed and the bacteria was washed with PBS (pH 7.4) thrice. Upon washing, the bacteria was re-suspended in HEPES Buffer (pH 7.4, 10mM EDTA) and incubated for 1 hour with EP-1, followed by washing with PBS trice and re-suspension of the bacteria sample in HEPES buffer before plated onto poly L-lysine coated glass slides and visualized on a confocal microscope under excitation wavelength $\lambda_{ex} = 670\text{nm}$.

In the negative control experiment, $OD_{600} = 0.5$ of bacteria cells suspended in Brain Heart Infusion (BHI) broth in an eppendorf tube was incubated with the addition of PBS instead of 3-Azido-D-Alanine at 37°C for one hour. After which, the medium was removed and the bacteria was washed with PBS (pH 7.4) thrice. Upon washing, the bacteria was re-suspended in HEPES Buffer (pH 7.4, 10mM EDTA) and incubated for 1 hour with EP-1, followed by washing with PBS trice to remove unbound reagents and re-suspension of the bacteria sample in HEPES buffer before plated onto poly L- lysine coated glass slides and visualized on a confocal microscope under excitation wavelength $\lambda_{ex} = 670\text{nm}$.

Selective bioimaging of AmpC Bla producing bacteria in mixed bacteria samples

Respective bacteria to be mixed were cultured overnight in BHI medium, harvested and suspended with PBS (pH 7.2) to obtain respective $OD_{600} = 0.5$ bacterial samples. The obtained bacterial samples were then mixed to obtain an $OD_{600} = 0.5$ *S. Aureus* – *E. cloacae* sample. This mixed bacteria sample was then incubated with 3-Azido D-Alanine at 37°C for one hour to allow for its metabolic uptake onto bacterial peptidoglycan. After which, the bacteria was washed with PBS trice, re-suspended in HEPES Buffer (pH 7.2, 10% EDTA) incubated with EP-1 for 1 hour. Upon incubation, the sample was washed trice with PBS to remove unbound reagents and re-suspended in HEPES Buffer (pH 7.2, 10% EDTA) before plated poly L-lysine coated glass slides and visualized on a confocal microscope under excitation wavelengths $\lambda_{ex} = 450\text{nm}$ and 670nm .

Peptidoglycan digestion and analysis

In order to validate and ensure bio-orthogonal covalent labeling of EP-1 onto bacterial peptidoglycan, analysis of peptidoglycan of bacteria cells was performed upon metabolic mediated labeling with EP-1. Cells were cultured to $OD_{600} = 0.6$ and incubated with 3-azido-D-alanine for 1 hour for its metabolic uptake on bacterial peptidoglycan. After which, the bacteria sample was washed trice with PBS before re- suspended with HEPES buffer (pH 7.2, 10% EDTA) and incubated with EP-1 for 1hr at 37°C for bio-orthogonal click reaction of the probe onto cell peptidoglycan. The bacterial sample was then washed trice with PBS to remove unbound reagents and re- suspended with HEPES buffer before incubation with chloramphenicol (130 $\mu\text{g/mL}$) for 15 min. The bacterial samples were subsequently centrifuged and washed with PBS to remove the antimicrobial agents. After removal of the agents, bacterial cells were subjected to

boiling water treatment for about 30 min. The cell extract was then centrifuged and the supernatant was further lyophilized. Finally, the lyophilized product was dissolved in 0.2 mL of water and pH was adjusted to 2.0 with 20 % phosphoric acid. The peptidoglycan lysates in the solution were analyzed by RP- HPLC monitoring the UV absorbance peak at 670 nm.

Flow cytometric Analysis

The overnight cultures of *DH5 α* , BL-21 and *E. cloacae* cells (10^8 CFU/ml) were incubated with were first treated with 3-azido-D-alanine for 1 hour for its metabolic uptake on bacterial peptidoglycan. After which, the bacteria sample was washed trice with PBS before re-suspended with HEPES buffer (pH 7.2, 10% EDTA) and incubated with EP-1 for 1hr at 37°C for bio-orthogonal click reaction of the probe onto cell peptidoglycan. Finally, the bacterial samples were washed with PBS trice, resuspended in HEPES buffer and the fluorescence of the cells was detected by a high sensitivity flow cytometer with a solid-state 650 nm continuous wave laser as excitation source. The emitted light was collected and split into two light paths for side scatter and red fluorescence detection respectively.

Cell Viability

The bacteria was suspended in PBS buffer solution with an OD₆₀₀ of 0.7 and diluted to a final concentration of 2×10^8 cfu/ml. 60 μ l aliquots of this suspension were mixed with D-Ala-Azide (0.1mM~2mM) and then added into the wells of the 96-well plate. After incubation for an hour, 25 μ l of each suspension of the bacteria were subsequently pipetted and plated onto LB agar plates then incubated at 37 ° C overnight. The number of colony forming units was counted after incubation.

In vivo zebra fish

All animal experimental procedures were performed in compliance with School of Chemical and Biochemical Engineering in Nanyang Technological University, Animal Care Committee. Wild type zebrafish (*Danio rerio*) were maintained following the standards of zebrafish care.

4.3. Results and Discussions

We first examined the sensitivity of EP-1 and EP-2 towards class A and class C beta-lactamases through the monitoring of changes in fluorescence emission upon purified enzyme activation in 0.1M, pH 7.4 Phosphate Buffer Saline (PBS). In the absence of beta-lactamases, EP-1 was shown to be relatively stable in PBS, with minimal fluorescence emission observed. In contrast, incubation of EP-1 with Class A TEM-1 Bla and Class C AmpC Bla up to 1 hour resulted in intense observed fluorescence emission with maximal emission at $\lambda_{em} = 710\text{nm}$. In particular, maximum fluorescence enhancement of ~ 150 fold was observed upon hydrolysis of EP-1 by AmpC Bla, while a fluorescence enhancement of ~ 45 fold was obtained upon TEM-1 Bla hydrolysis. (Fig 4.1a, b).

Figure 4.1 (a) Emission spectra of EP-1 and (b) EP-2 (10 μM) before and after incubation with 50 pM of TEM-1 and AmpC Blas in PBS (pH = 7.4).

Incubation of EP-2 with TEM-1 and AmpC Bla similarly yielded favorable results with maximum fluorescence enhancement of ~150 fold observed upon both TEM-1 and AmpC Bla hydrolysis. These results obtained indicates clearly the sensitivity of both EP-1 and EP-2 towards Bla hydrolysis and demonstrates the viability towards the monitoring fluorescence emission of both probes as an indicator of Bla presence, due to successful expulsion of the conjugated dark quencher moiety. Yet, the stronger fluorescence enhancement obtained upon AmpC hydrolysis as compared to TEM-1 hydrolysis also demonstrates that EP-1 is able to recognize AmpC Bla. It further proves that that the presence of a steric bulk group (DBCO) on 7' amino end of the beta- lactam ring enables the modified cephalosporin's to be more susceptible and selective towards Class C Bla. In order to prove that hydrolysis of EP-1 and EP-2 was solely due to the presence of beta-lactamases, EP-1 was also incubated with TEM-1 Bla and AmpC Bla with respective Class A and C inhibitors Clavulanic acid (CA) and Aztreonam (AZT). In the presence of these inhibitors, the catalytic efficiencies of TEM-1 and AmpC Bla were severely diminished. Comparisons of maximal fluorescence obtained shows significant reductions in fluorescence intensity upon TEM-1 Bla and AmpC inhibition. (Fig 4.2.), establishing that Blas are indeed necessary for probe hydrolysis and fluorescence visualization.

Figure 4.2. Fluorescence intensity of EP-1 in the presence and absence of AmpC Bla inhibitor (AZT) and TEM-1 inhibitor (CA)

To better investigate results obtained for EP-1 and EP-2 hydrolysis by TEM-1 and AmpC Blas. Enzyme kinetics analysis was also conducted for EP-1 in PBS (pH 7.4) with fluorescence emission changes measure over a period of 1 hr. (figure 4.3) The results obtained shows that EP-1 could indeed be hydrolyzed at reasonable efficiencies by TEM-1 and AmpC Bla with obtained catalytic constants ($K_{cat} = 29.2 \text{ min}^{-1}$, 14.3 min^{-1}) and Michaelis constant ($K_m = 15.1 \mu\text{M}$, $12.1 \mu\text{M}$) denoting increased hydrolysis rates of EP-1 by AmpC over TEM-1 Bla. In comparison, Enzyme kinetics analysis performed on EP-2 for TEM-1 and AmpC resulted in catalytic constants ($K_{cat} = 16.1 \text{ min}^{-1}$ and 17.2 min^{-1}) and Michaelis constant ($K_m = 11.1 \mu\text{M}$, $12.3 \mu\text{M}$) similar in magnitude as compared to EP-1. These results demonstrated that EP-1 is able to recognize Class C Blas due to the presence of the bulky DBCO moiety as compared to EP-2, complementing previous fluorescence emission results obtained in the enzyme triggered assays.

Figure 4.3. Enzyme kinetics of (a) EP-1 and (b) EP-2 (0~100 μM) after 1 hr incubation with AmpC and TEM-1 Bla respectively.

Inspired by the *in vitro* assay results, we further investigated the intracellular uptake and

enzyme-triggered covalent localization of EP-1 in pathogenic bacteria. In this study, two different groups resistant bacteria strains were chosen. The first group contain bacteria species includes *Enterobacter cloacae* (*E. cloacae*), owing to its high expression of Amp C and the second group as a positive control which has a high expression of TEM-1, *E.coli* BL21. Additionally, bacteria without Bla expression that were antibiotic susceptible, DH5 α were chosen as negative control. Furthermore, a cell viability test was carried out to validate the toxicity of D-Ala-Azide towards the resistant bacteria (Figure 4.4). As shown in the figure, the toxicity of the D-Ala-Azide shows relatively low toxicity towards the pathogens at low concentration (0.1mM ~2mM). This makes D-Ala-Azide a suitable candidate for metabolic labeling onto the bacteria surface. The N₃ -tagged peptidoglycan could be introduced into these resistant strains through intrinsic metabolism by simply feeding the D-Ala-N₃ precursor into the culture medium. After the successful incorporation of the D-Ala-N₃ to the peptidoglycan, a copper-free click reaction based on DBCO-Cy3 was chosen to perform a copper-free click bioorthogonal linkage to the D-Ala-N₃ treated bacteria strains. Subsequently, the covalent labeling on the bacteria membrane was monitored by confocal microscopy. In figure 4.5, the bacteria fed with D-Ala-N₃ (1mM), a bright yellow fluorescence was observed on the surface of the peptidoglycan while the control experiments, which consist of strains without the pretreatment of D-Ala-N₃ shows no obvious fluorescence. This result suggests the high feasibility of covalent conjugation of functional moieties on the surface of the peptidoglycan through native metabolic biosynthetic pathway.

Figure 4.4. Bacteria viability pre-incubated with D-Ala-Azide (0.1mM ~ 2mM)

Figure 4.5. Confocal imaging of N₃-tagged *Enterobacter cloacae* (*E. cloacae*), BL-21 and DH5α, with Dibenzocyclooctyne Cy3 (DBCO Cy3) respectively. Yellow: Cy3 (λ_{ex} 550/50 nm, λ_{em} 580/50 nm).

Furthermore, to show the applicability of the probe to be selectively covalent labeled on the surface of the resistant bacteria strain with high expression of AmpC, the following experiments were conducted. Typically, two sets of experiments which

contain same group of bacteria was carried out; one set was metabolized with D-Ala-Azide while another as a negative control with the absence of D-Ala-Azide in the culture medium. These two bacteria sets were separately incubated with 10 μ M of EP-1 for 30 minutes at 37 °C, and subsequently subjected to confocal microscopy imaging. As shown in figure 4.6, an obvious strong red fluorescence emission observed after the incubation of EP-1 with AmpC expressed N₃-tagged *E. cloacae* while the non-metabolized bacteria show weak fluorescence. The control with high TEM-1 expression BL-21 (N₃-tagged and non-N₃-tagged) were observed with minimum signals obtained 670nm. These results clearly indicated that the N₃-tagged bacteria, *E. cloacae*, which contains high expression of AmpC enables the covalent localization of the probe on the surface of the pathogenic bacteria, prevents the diffusion of the fluorescent probe after multiple washings unlike the bacteria without N₃-tagged. Importantly, there was no obvious fluorescence detected in *E. cloacae* pre-treated with AmpC inhibitor, Aztreonam (AZT) and positive control BL-21 pretreated with CA(Figure 4.7).

Figure 4.6. (a) Fluorescent imaging of EP-1 (10 μ M) in metabolized and non-metabolized bacteria (b) Fluorescence and phase-contrast microscopy imaging of *E. cloacae* wild type cells labeled with EP-1 (10 μ M).

Figure 4.7. (a) Fluorescent imaging of EP-1 (10 μ M) in metabolized bacteria with inhibitor AZT (*E.cloacae*) and CA (*E.coli* BL-21)

Furthermore, we surveyed the feasibility of quantifying the specific labeling of AmpC expressing resistant bacteria using flow cytometry (FCM), figure 4.8. In this study, 3 different bacteria strains (*E. cloacae*, BL-21 and DH5 α with and without metabolized) were separately used and treated with EP-1 (10 μ M) for 30 mins at 37 $^{\circ}$ C and the fluorescence signals were collected at 670 nm. A strong fluorescence signal was detected ($\sim$ 10 folds) for EP-1 after incubation with AmpC expressed *E. cloacae*. On the contrary, a much weaker fluorescent enhancement was observed ($\sim$ 2 folds) for TEM-1 expressed BL-21 as compared to the negative control DH5 α bacteria strain. The obtained data clearly indicated that EP-1 could be used to serve as a reliable reporter for quantifying the AmpC activity in pathogenic bacteria.

Figure 4.8. (a) Flow cytometry analysis of EP-1 (10 μM) with 3 different N₃-tagged bacteria (b) non-N₃-tagged bacteria

Inspired by the promising selective imaging results in resistant bacteria and flow cytometry, we further investigated the possibility of the probe to be applied directly on zebrafish infected with pathogenic bacteria. Typically, we microinjected *E. cloacae*, BL-21 and DH5α separately into the yolk sac of the zebrafish larvae at 72 hours post- fertilization (hpf). Upon successful infection, the larva was then bathed with D-Ala- azide for 1 h to metabolically incorporate azide handle on the surface of the bacteria. In order to study the feasibility of the metabolic peptidoglycan labeling for the localization of EP-1, the larvae were further incubated with EP-1 for 1 h to allow effective covalent click reaction onto the N₃-tagged bacteria in the zebrafish (n =9). Fluorescence imaging of the probe-incubated larvae infected with the N₃-tagged *E. cloacae* shows significant fluorescence signals observed at 710 nm throughout the zebrafish (Figure 4.9) while zebrafish infected with N₃-tagged BL-21 and DH5α shows relatively weaker fluorescent. More significantly, separate control experiments with N₃-tagged bacterial infections without EP-1 incubation, as well as non-azide labeled bacteria with probe incorporation exhibits minimum fluorescent signals in contrast (Figure 4.10). Upon 72 h clearance, the obvious fluorescence in zebrafish with N₃- tagged *E. cloacae* treated with EP-1 could still

be observed, on the other hand, only a weak fluorescence signal was detected for other controls. Such results obtained demonstrate that EP-1 labeling could localize onto bacteria surface and sense pathogenic bacteria with high expression of AmpC *in vivo*.

Figure 4.10 *In vivo* fluorescent imaging of EP-1 in zebra fish

Currently, most studies have been focused in the area of Class A Bla and the systematic studies for the selective localization of Class C Bla for *in vitro* and *in vivo* setting is under exploited. This makes precise clinical diagnosis difficult for accurate prescription. One of the critical needs for improving the diagnosis of specific types of bacterial infection is the use of appropriate biocompatible reactions that can selectively localize in this antibiotic resistant bacterium. So far, fluorescence imaging has been regarded as a widely-used technique for bioimaging of intrinsic cellular and understanding the endogenous biochemical reactions. One of the key benefit is its high sensitivity and function as a rapid sensing tool. However, despite these advantages, most commercially available fluorophores may suffer the consequences of free diffusion at the active site of the enzyme leading to a decrease in fluorescence signal.

Despite covalent labeling approaches heralded as a means of mitigating such a shortfall, early designs of biocompatible reaction probes have shown to encompass problems such as poor labeling efficiency and specificity. In that regard, metabolic labeling represents a robust concept for the efficient and localized tagging of probes and other molecules onto specific cellular regions. The high specificity, selectivity and ease of use presents great qualities for its use not just for the greater understanding of metabolic processes in resistant bacteria, but also serves as a viable platform for the sensing of resistant bacteria in biomedical settings.

In this study, we present a simple and selective approach towards covalent localization of EP-1 on the surface of the bacteria in living cells and *in vivo*. EP-1 not only is able to localize onto resistant bacteria, but also was capable of selectively fluorescing upon hydrolysis by AmpC enzyme. This can be attributed to the steric bulk of the DBCO group conjugated onto the 7' amino end of the cephalosporin-based probe, which prevents effective hydrolysis by TEM-1 Bla but allows for probe activation by AmpC. Such significant property confers many advantages for accurate medical diagnosis during the treatment process. During the live cell imaging process, only pathogenic bacteria, which express AmpC, would induce EP-1 hydrolysis, triggering the expulsion of the dark quencher moiety on the 3rd position of the cephalosporin, BHQ-3. The minimization of FRET Quenching thus allows visualization of strong fluorescence emission at 710 nm. In contrast, resistant bacteria with TEM-1 expression reveal minimum fluorescence signal²⁵⁻²⁸.

Furthermore, by exploiting the advantage of the endogenous metabolic process in pathogenic bacteria, we have incorporated an azide handle on the surface of the bacteria through their metabolic biosynthetic pathway. This would allow the bio- orthogonal DBCO group present in EP-1 to perform a copper-free click covalent reaction, therefore

ligating and localizing EP-1 onto cell peptidoglycan. More importantly, unlike most traditional fluorescent probes based on Bla hydrolysis, the metabolic probe used in this study can help to minimize the diffusion of the activated probes in living tissues. In this way, it represents a more reliable method for rapid observation and differentiation of clinical resistance especially for systematic investigations of individual cells in a mixed population²⁹⁻³⁰. By taking this promising advantage, we have carried out a series of experiment on two separate sets of groups of bacteria, one set contain metabolized bacteria while the other cultured in the absence of D-Ala azide, and systematically compared the differences for covalent localization study *in vitro* and *in vivo*. Our data clearly revealed that the AmpC expressing N₃-tagged bacteria has a stronger fluorescence signal for both *in vitro* and *in vivo* studies. This result suggests that the metabolized bacteria enables covalent localization on the surface of the bacteria and prevents the fate of the hydrolyzed probe from diffusing away from the bacteria surface. Moreover, the process of effective localization of EP-1 would not result in significant cell death or tissue damage, thus provides as a promising candidate for clinical application in bacterial infections. Furthermore, *in vitro* and *in vivo* studies have also demonstrated that such unique and selective AmpC Bla responsive cephalosporin based probe could target a specific class of bacteria in a mixed population, which could allow for practical applications in a hospital environment.

4.4 Conclusions

In summary, we have developed a novel targeting strategy based on cephalosporin backbone to be covalent localize on AmpC expressed N₃-tagged bacteria by taking advantage of the installation of a bulky DBCO at its 7th position. A NIR fluorescent probe, EP-1, enabled the successful detection and imaging in *in-vivo* bacterial infections models such as zebrafish through a copper-free click reaction. More detailed

experimental works are required be carried out to verify the application in animal study. One of the important future work is the study of the toxicity of the probes before it is further carried out into animal study. This well-defined rational design could be readily and easily extended into other platforms and open new doors for future clinical applications.

4.5 References

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Chapter 5

Enzyme Responsive Fluorogenic Probe for Specific Localization and Imaging of AmpC Expressed Resistant Bacteria

5.1 Introduction

Since the last century, β -lactam antibiotics such as cephalosporin and penicillin's have been developed as one of the major class of antimicrobial agents for treating bacterial infections in clinical settings¹⁻³. And among most of the resistant enzymes that have been secreted by the bacteria, β -lactamases (Bla) is one of the most common ones that have been linked to various infectious diseases⁴⁻⁹. Between the different classes of Bla, Class A and C are the most significant members responsible for hydrolyzing the β -lactam rings and render the treatment effects by the commercially available antibiotic such as penicillin. Class A Blas has been well studied and investigated for imaging biological process as well as resistance inactivation⁵. However, Class C Bla is less understood and investigated despite its extensive resistance, which poses a threat to the health community. Therefore, the establishment of effective strategies for detecting and bioimage of such resistant bacteria expressing Class C Bla is of high importance.

To date, there are numerous laboratory-based methods to detect such resistance bacteria⁷⁻¹², and among them optical imaging for effective monitoring resistant bacteria has shown great potential. This method of imaging has been greatly extended and utilized in various biomedical applications¹³⁻¹⁵. The use of fluorescent imaging via small molecules probes, fluorescent proteins and metabolic uptake represents an established method for monitoring of resistant bacteria *in vitro* and *in vivo* studies. Yet, this approach remains limited towards monitoring the dynamic process within the native environment in living cells owing to diffusion of small molecule probes upon enzyme activation and the potential of perturbing the endogenous properties of the enzyme.

Therefore, the rational design of a molecular probe with tractability and immobilizing properties for real-time imaging of resistant bacteria with Class C Bla expression is highly desirable. One application is the use of long lipid chain that could confine the probe into the lipid compartments of bacteria cell membranes, thereby increasing the effective probe-enzyme interactions⁴². Furthermore, fatty acid lipid molecules are naturally expressed cell surface components that are well recognized as integral membrane structures with a close relation to cell wall stiffness and susceptibility to various cell types of cell function. Unlike genetic engineering and metabolic uptake, the process of lipid insertion into the cellular membrane of the bacteria, would not alter the endogenous biological properties of the bacteria. Importantly, the insertion of the lipid carbon chain would help in the localization of the probe onto the surface of the bacteria; therefore minimize the diffusion of the activated probe from the active sites. In this manner, lipid insertion can increase the probe to enzyme interaction, minimizing the effects of diffusion and reducing any possible metabolic uptake process which might perturb the endogenous properties of the proteins.

5.1 Design and Methods

Herein, we present a small molecule probe that can selectively monitor Class C Bla, AmpC, and anchor to the surface of the resistant bacteria (Scheme 5.1). A bulky functional group, dibenzocyclooctyne (DBCO), would be installed onto the 7' position of the cephalosporin molecule making the molecule more selective to Class C Bla. This is evidently due to the larger bulky group, which makes it incapable to fit into the binding pocket of Class A Bla. In order to conduct real-

time imaging of the molecular probe, the cephalosporin's was flanked with an activatable FRET pair consisting of a fluorescein (FIT) and a quencher Dabcyl (4-(dimethylaminoazo)benzene-4-carboxamide)^{8,28,42}. After the insertion of the lipid chain onto the surface of the bacteria, the fluorescent probe would be conjugated with the azide functional group on the lipid surface via a copper-free click reaction. Upon hydrolysis by Class C Bla, the change in fluorescence signal would reflect the biological activities of AmpC²⁸. Moreover, to effectively immobilize the molecular probe onto the bacteria surface, fatty-acid lipid moieties of different carbon length would be carefully screened. The optimized lipid moiety would serve as a very effective targeting vector to help localizing the probe onto the surface of the bacteria (Scheme 5.1). In this way, the drug molecule will be more sensitive and selective towards AmpC expressed antibiotics resistant bacteria. In order to examine our hypothesis, we first synthesized the desired MPA as accordingly:

Scheme 5.1. Enzyme Responsive Molecular Probe A (ERMPA) activation by Blas after localization of lipid chain on the surface of resistant bacteria.

Synthesis of 2

NH₂-MTT-lysine-Boc (2.01 mmol, 495.6 mg) and triethylamine (8.01 mmol, 1.20 mL) were added to a solution of 4-MttCl (4.00 mmol, 1170.0 mg) in 1:2 DMF/CH₂Cl₂ (3.00 mL) and was allowed to stir vigorously for the overnight. The reaction mixture was then quenched with 3.00 mL of methanol, and dried under rotatory evaporation. Next the mixture was then washed with ethyl acetate (EtOAc), water and finally with brine. The organic layer was subsequently dried over anhydrous magnesium sulfate and concentrated under rotatory evaporation. The crude mixture was then purified with flash chromatography (EtOAc: hexane) to afford a white solid product 501mg, 59.0%. ¹H NMR (CDCl₃, 300 MHz): δ7.10-7.41, (15H, m), 2.36 (3H, s), 2.10 (1H, s), 1.70 (1H, brs) 1.56 (3H, brs), 1.40 (9H, s), 1.31 (4H, t, *J* = 7.0 Hz).

Synthesis of 4

Tritylchloride (4.00 mmol, 1142.2 mg) was added into 2.50 mL of DCM and next 4-aminothiophenol **3** (4.40 mmol, 574.8 mg) was added. Finally, 1ml of TFA was added into the reaction mixture in a single portion and allowed to stir for 3 hours. After monitoring the starting material has been consumed in the reaction, the reaction was allowed to concentrate under reduced pressure and then purify using flash chromatography to afford a yellow-brown solid 381mg, 50%. ¹H NMR (CDCl₃, 300 MHz): δ7.18-7.42 (15H, m), 6.81 (2H, d, *J* = 8.0 Hz), 6.40 (2H, d, *J* = 8.0 Hz).

Synthesis of 5

DabcyI COOH (0.30 mmol, 91.4 mg) was added into a mixture of DCM and pyridine in a 1:1. Follow by EDC.HCl (2.00 mmol, 380.7 mg) were then added and it was allowed to stir for 10 minutes at ambient temperature. Next **4** (0.300 mmol, 128.8 mg) were added and subsequently a catalytic amount of 4-DMAP (0.0175 mmol, 2.2 mg) was added into the reaction mixture and allowed to stir vigorously for the next 3 hours. The reaction mixture was allowed to be concentrated and extracted with EtOAc, sodium bicarbonate (NaHCO₃), brine and finally it was dried over with anhydrous magnesium sulphate. The crude mixture was then purified using column chromatography to afford an orange crystals 71mg, 46%; ¹H NMR (CDCl₃, 300 MHz): δ7.90-7.95 (6H, m), 7.75 (1H, s), 7.42-7.45 (6H, m), 7.35 (2H, d, *J* = 8.7 Hz), 7.18-7.28 (8H,m), 7.00 (2H, d, *J* = 8.5 Hz), 6.81 (2H, d, *J* = 9.0 Hz), 3.10 (6H, s).

Synthesis of 8

ALCH.HCl **1** (1 mmol, 451.0 mg) was added into CH₃CN/dioxane (1:1) and then 198 μL of triethylamine was added dropwise into the mixture to form a colourless solution. Subsequently, **2** was added (1 mmol, 459 mg), HOBt (1.30 mmol, 185.1 mg), and EDC.HCl (2.00 mmol, 451.2 mg) were added in a single portion. The reaction mixture

was allowed to stir at room temperature overnight. After the reaction mixture was concentrated under reduced pressure, it was purified with flash column to afford a white solid of 501mg, 61%. ^1H NMR (CDCl_3 , 300 MHz): δ 7.01-7.55 (25H, m), 5.80 (1H, dd, $J_1 = 4.0\text{Hz}$, $J_2 = 9.0\text{ Hz}$), 5.00 (1H, d, $J = 5.2\text{ Hz}$), 5.30 (1H, s), 4.92 (1H, d, $J = 7.0\text{ Hz}$), 4.40 (2H, dd, $J_1 = 6.8\text{ Hz}$, $J_2 = 12.0\text{ Hz}$), 3.50 (2H, dd, $J_1 = 18.0\text{ Hz}$, $J_2 = 6.80\text{ Hz}$), 2.10 (2H, t, $J = 6.8\text{ Hz}$), 2.32 (2H, s), 1.74-1.84 (2H, m), 1.50-1.59 (4H, m), 1.43 (9H, s).

Synthesis of 10

5 (0.110 mmol, 71.9 mg) was added into 3.00 mL of CH_2Cl_2 , next 5.0 μL of triisopropylsilane was added in a single portion to the reaction mixture. Finally 1ml of TFA was added dropwise into the mixture and was allowed to stir under ambient temperature for 1hr. After concentrating the reaction under reduced pressure, the mixture was then allowed to be vacuum dry for 3 hours to form a red solid **6**. The crude product of **6** was then added into 3.00 mL of DMF, 25 2,6-Lutidine μL was added into the reaction mixture along with **8** and with a catalytic amount of sodium iodide. After the reaction has stirred overnight, the crude mixture was then extracted with ethyl acetate, water and brine and finally dry over with anhydrous magnesium sulphate. The crude mixture was finally purified with column chromatography to form an orange solid of Orange solid; yield 110mg, 63%; MS: 1238.73, 619.37.

Synthesis of 12

10 (0.0800 mmol, 100.0 mg) was dissolved into 1.00 mL of DCM and then 20.0 μ L of TFA was added drop wise into the reaction mixture and stir for 4 hours. The mixture was then concentrated under reduced pressure to form **11**. Next the crude mixture obtained was dissolved in 0.5ml of DMF and follow by the addition of 23.4mg of FITC. Finally, triethylamine was added into the reaction mixture and allowed to be stirred overnight. After monitoring the reactant has been consumed in a thin layer chromatography, the mixture was extracted with ethyl acetate, water, brine and finally dried over with anhydrous magnesium sulphate. The crude was then finally purified using flash column to form an orange colour solid. MS: 1373.29, 686.65.

Synthesis of ERMPA

12 (0.00552 mmol, 9.0 mg) in 300.0 μ L of DCM at 0°C, next 50.0 μ L of TFA and 10.0 μ L of TIPS was then added into the mixture and stirred continuously for 4 hours at room temperature. The Crude mixture was then dissolved in 200.0 μ L of DMF, followed by addition of DBCO–NHS (0.2 mmol, 8.4 mg) and DIEA (0.0280 mmol, 4.8 μ L). After stirring the reaction mixture overnight at room temperature, the reaction was left to stir overnight and purified using reverse phase high performance liquid chromatography to form an orange solid. MS: 1393.58, 696.79.

Synthesis of C6 carbon lipid

Aryl chloride (10.00 mmol, 1.40 mL) in 30.00 mL of DCM was added ethanolamine (15.00 mmol, 0.92 mL) dropwise, followed by 3.00 mL of TEA dissolved in 30.00 mL of DCM. The reaction mixture was allowed to stir for 1 hour and then it was allowed to concentrate under reduced pressure and purified with flash column to form a white solid C6 carbon lipid 1.01g 75%; ¹H NMR (CDCl₃, 300 MHz): δ 3.62 (2H, t, J = 5.1 Hz), 3.32 (2H, m), 2.18 (2H, t, J = 8.0 Hz), 1.58 (2H, m), 1.26 (4H, m), 0.85 (3H, t, J = 6.2 Hz).

Synthesis of C6 carbon lipid N₃

C6 carbon lipid (1.00 mmol, 160 mg) in 15.00 mL of DMF was heated to 120°C and added 120 μ L of DBU, followed by 160 μ L of azide. The reaction was allowed to stir for 3 hours, then it was further diluted with diethyl ether. The crude was then extracted with ethyl acetate, water and brine and finally dry over with anhydrous magnesium sulphate. The crude product was then purified with flash column to form a white solid with 400mg, 45%. ¹H NMR (CDCl₃, 300 MHz): δ 3.40 (4H, m), 2.18 (2H, t, J = 7.5 Hz), 1.55 (2H, m), 1.30 (4H, m), 0.90 (3H, t, J = 6.8 Hz).

Click Chemistry of ERMPA with C₆ Carbon Lipid Azide

C6 carbon lipid (0.001 mmol, 0.16 mg) was dissolved in DMSO: H₂O (1:9) and was added into the ERMPA (0.001 mmol, 1.5mg) and stirred at room temperature for an hour. The crude product was analyzed with LC-MS (Figure 5.1). MS: 1578.18

Figure 5.1: a) HPLC analysis of clicked product of ERMPA with C6 lipid azide b) LC-MS analysis of ERMPA with C6 carbon lipid azide.

Scheme 5.1: Synthetic Route for the synthesis of ERMPA (Molecular Probe A)

Enzyme Hydrolysis Assay:

Reaction mixtures (100 μ L each) containing molecular probe A (10 μ M) were incubated separately with 50 nM of AmpC and TEM-1 respectively in 0.1 M PBS buffer (pH 7.4) at 37 $^{\circ}$ C for 1 hr and subjected to fluorescent measurement. In control experiments, molecular probe A was incubated with PBS buffer at 37 $^{\circ}$ C for 1 hr. To study the enzyme specificity, the enzyme, TEM-1 and AmpC is pretreated with inhibitors CA and AZT (100 μ M) respectively and with molecular probe A for fluorescent studies.

Click Chemistry of Probe A with Lipid Azide

Typically, the lipid azide (10 μ M). was incubated with the overnight culture of bacteria suspension were diluted to 10^8 cells/ml for 30 minutes. The bacteria was washed with PBS solution 3 times and followed by incubated with molecular probe A (10 μ M). Bacteria imaging tests were then conducted with Zeiss LSM 800 confocal microscope.

Confocal Microscopic Bacteria Imaging

The overnight culture of bacteria suspension was diluted to 10^8 cells/ml and incubated with molecular probe A (10 μ M). After washing the PBS, the bacteria cells were spotted on poly-L-lysine pretreated glass slides and immobilized with coverslips. Bacteria imaging tests were conducted with Zeiss LSM 800 confocal microscope. To study the enzyme specificity, the bacteria were pretreated with enzyme inhibitor CA and AZT and labeled with molecular probe A for imaging studies.

5.3 Results and Discussions

The enzyme responsive molecule probe A (ERMPA) is first synthesized by conjugating a bulky DBCO group on to the 7' position group of cephalosporins. Subsequently, a

fluorophore fluorescein isothiocyanate (FITC) was further covalently linked to the N-terminal of the lysine functional group with an effective quencher molecule Dabcyl conjugated on the 3' position of the 4-aminothiophenol. Thus, the fluorescence of FITC would be quenched upon excitation at 470 nm and minimum fluorescence should be observed. In order to test the selectivity of the ERMPA, typically we incubated the 10 μ M probe in 10% of phosphate buffer solution (PBS) at pH = 7.4 in 37 °C. Subsequently, AmpC was added into the mixture and further incubated for 1 hr. As shown in figure 5.5, the fluorescence intensity was enhanced by approximately 80 times after incubation with AmpC as compared to the negative control in the absence of Bla. A similar study was then carried out using TEM-1, it was observed the fluorescence intensity was increased but at a lower fold enhancement of about 20. Furthermore, to examine if the fluorescence enhancement is a result caused by the Bla activity, we performed another series of inhibitor experiment. Briefly, the Bla enzyme, AmpC and TEM-1 is first pretreated with their respective inhibitor, AZT and CA, at 100 μ M before the incubating with ERMPA. The result shown in Figure 5.2 demonstrated a minimum fluorescent enhancement for both TEM-1 and AmpC. The result clearly demonstrated that the serine-70 in the active site of Bla is capable to hydrolyze the ERMPA which resulted in the release of the quencher Dabcyl leading to the enhancement in fluorescence. The lower fluorescence intensity enhancement as observed in TEM-1 was likely due to the presence of the bulky DBCO functional group, which slows down its rate of hydrolysis. In comparison to the higher fluorescence intensity as observed by AmpC, is due to the presence of bigger binding active site that facilitated to a more rapid hydrolysis to the beta-lactam ring.

Figure 5.2. Enzymatic hydrolysis of ERMPA with TEM-1 and AmpC as in PBS (pH = 7.4).

Delighted by the positive results obtained in our buffer enzyme hydrolysis assay, we further investigated the applicability of the ERMPA to be used in live bacteria cells to selectively monitor AmpC Bla activities. Generally, we have selected 3 types of Gram-negative bacteria: *E.coli* DH5 α , *E.coli* BL-21 and *Pseudomonas aeruginosa*. These bacteria were chosen owing to the high expression of Class A Bla, TEM-1 for *E.coli* BL-21 and AmpC in *Pseudomonas aeruginosa*. The negative control *E.coli* DH5 α was used in the study, as it is susceptible in the presence of antibiotics. The cells are typically cultured using LB-broth medium overnight and experiment was performed in an OD₆₀₀ = 0.5. The cultured bacteria was then incubated in 10% of phosphate buffer solution (PBS) at pH = 7.4 at 37 °C and 10 μ M of ERMPA was added into the buffer solution containing the bacteria for an hour. The treated bacteria were then subjected to confocal microscopy and the result is as shown in figure 5.3.

Figure 5.3. Confocal microscopy imaging of resistant bacteria with ERMPA (10 μ M) in PBS for 1hr, pH = 7.4. ERMPA: Ex = 400/50 nm; Em = 500/50 nm.

The result shown that there is a strong fluorescence enhancement in the AmpC expressed *Pseudomonas aeruginosa* while minimum fluorescence was observed in both *E.coli* BL-21 and *E.coli* DH5 α . Clearly, molecular probe A is able to demonstrate a higher selectivity towards AmpC expressed *Pseudomonas aeruginosa* as compared to TEM-1 expressed *E.coli* BL-21 and the negative control *E.coli* DH5 α ¹⁷. To examine the feasibility of the ERMPA could perform localization onto the surface of the bacteria in the presence of the lipid molecule. We further carried out a series of experiments by examining different lipid lengths that may assist in the localization of the probe onto the surface of the bacteria. In this study, we screened a series of lipid molecules conjugated with ERMPA to validate our earlier hypothesis that the lipid-based cephalosporin's enables the localization of the probe on the bacteria surface,

thus increasing enzyme-probe interactions. The first preliminary result is revealed in Figure 5.4. As shown in the figure, strong fluorescence emission was observed after incubation of lipid based ERMPA with AmpC expressed *Pseudomonas aeruginosa* whereas similar bacteria incubation with TEM-1 expressed BL-21 led to only weak fluorescence. Importantly, there was no obvious fluorescence detected in the control *E.coli* DH5 α and a more significant fluorescence enhancement was observed in the resistant bacteria after ERMPA clicked with the lipid azide. These results unequivocally indicated the intrinsic capability of the rationally developed ERMPA to selectively localize and report AmpC activities in resistant bacteria.

Figure 5.4. Confocal microscopy imaging of (a) resistant bacteria with ERMPA (10 μ M) in PBS, pH = 7.4 (b) Magnified view of the ERMPA clicked with lipid onto the surface of bacteria (TEM-1: *E.coli* BL-21 and AmpC: *Pseudomonas Aeruginosa*). ERMPA: Ex = 400/50 nm; Em = 500/50 nm.

5.4 Conclusions

In this study, we constructed an enzyme responsive probe conjugated with lipid, which can very effectively localize on the surface of the bacteria. The cephalosporin's probe conjugated on the lipid surface could be efficiently confined and localized on the surface of the Class C expressed resistant bacteria³⁹⁻⁴¹, thereby increasing the effective probe-enzyme interactions. Despite the initial success, more experiments must be carried out to screen various lengths of lipid chains and optimize the localization of the ERMPA onto the surface of the bacteria. After the optimization of the probes, the toxicity of the ERMPA and the various length of lipids would be carried out for *in vitro* and *in vivo* studies. In this manner, we believe that such unique probes present promising advantages compare to metabolic uptake offers an attractive approach for labeling resistant bacteria without the need of artificial metabolic uptake or genetic engineering, which can be applied to biomedical applications in antibiotic resistance pathogens.

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Summary and Perspective

The wide spread of bacteria resistant problem has called an immediate attention in the biomedical world to investigate and provide a precise diagnosis method for the types of infections caused. One of the most commonly known infections in bacteria is the secretion of beta lactamase, which destroyed the beta lactam ring for the proper treatment process. Among the various classes of beta lactamases, A and C are particularly the most prevailed. Therefore, development of probes that is capable to recognize such resistant enzymes become increasingly important for proper treatment.

Chapter 2 presented the construction of a molecular fluorogenic probe based on aggregation-induced emission (AIE) with the installation of a bulky ATMO group at the 7th position of the cephalosporin. This will enable the selective recognition of the AmpC expressed resistant bacteria. This study will be very useful to study the resistant mechanism in AmpC expressed bacteria as well as a rapid way to monitor such pathogenic bacteria in hospital setting.

In chapter 3, the synthesis of UCNs based cephalosporin fluorogenic probe for intracellular imaging of gene expression beta lactamase in cells. In this study, the strategy of activatable imaging with FRET effect is employed, where the endogenous resistant enzyme is used as the target reporter. By combining the use of upconversion

nanoparticle and FRET, this method enables the monitoring of the activities of gene-reporter cells expressing Bla enzyme at higher wavelength with lower photo-damage and better tissue penetration.

In chapter 4, the study presented the synthesis and construction of a metabolic probe for the selective covalent localization of the probe on AmpC expressed pathogenic bacteria. This study combined the use of metabolizing the resistant bacteria with D- Ala-Azide and through of click chemistry using a bulky DBCO functional group at the third position of cephalosporin's, which can selectively recognize AmpC expressed pathogens.

In the chapter 5, a study based on an enzyme responsive probe conjugated with lipid, which can very effectively localize on the surface of the bacteria, has been developed. In this study, the cephalosporin's probe conjugated on the lipid surface could be efficiently confined and localized in the lipid compartments of the bacteria cell membrane, thereby increasing the effective probe-enzyme interactions. Such unique probes indicated promising advantages compare to metabolic uptake offers an attractive approach for labeling resistant bacteria without the need of artificial metabolic uptake or genetic engineering that may potentially perturb the endogenous properties of the bacteria.

In conclusion, these probes have been successfully constructed and used for the detection and bioimaging of pathogenic bacteria expressing Bla, in particular AmpC Bla. The construction of such probes greatly dependent on the molecular basis for biological events to ensure the signal is specific to Bla activity and through the

combination of pre-quenching enables a higher sensitivity. These functions will provide as a powerful tool for microbiology and various biological study.

Future work

Drug resistance is a serious problem in antibacterial therapies, which may potentially cause a major threat to public health. To address this worsening problem, there calls an urgent need for the development of new strategies against bacterial pathogens. One of the future work towards circumventing drug resistance problem against bacteria is the design of new probes that enables the selective metabolic uptake¹ of D-Ala-Azide to the resistant bacteria expresses Class C Bla, AmpC. After the successfully labeling of the azide on the surface of the bacteria, it would undergo a copper-free click reaction with DBCO-Cy5. In this manner, it will reduce any possible background fluorescence signals caused by non-resistant bacteria. This model can be further carried out into both *in vitro* and *in vivo* settings. Toxicity test would also be carried out to ensure the probes are suitable for application to animal model.

Moreover, more investigations can be made in other forms of resistant enzymes present in bacteria pathogens such as carbapenemase^{2,3}. Carbapenemase has been widely regarded in the medical world as one of the enzyme responsible for numerous bacteria infectious diseases. One of the methods to accurately detect this resistant strain enzyme is to modify the stereo configurations on the 7th position of the cephalosporin of the ring from an R to S configuration^{4,5}. Through this method of modification to the core structure of the beta lactam ring, it will be highly sensitive to Carbapenemase as compared to Bla. A FRET based moiety can be constructed with the 7th position modified to S configuration for the detection of Carbapenamase

enzymes after the expulsion of the acceptor pair, which leads to a change in fluorescence. This will enable the effective monitoring of Carbapenamase activities.

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