Novel antibacterial therapy : mechanical force and light-activation

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**NANYANG
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**NOVEL ANTIBACTERIAL THERAPY – MECHANICAL
FORCE AND LIGHT-ACTIVATION**

DU QUANCHAO

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2021

NOVEL ANTIBACTERIAL THERAPY – MECHANICAL FORCE AND LIGHT-ACTIVATION

DU QUANCHAO

**SCHOOL OF PHYSICAL AND MATHEMATICAL
SCIENCES**

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for
the degree of Doctor of Philosophy

2021

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I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal in which I am listed as an author.

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The contributions of the co-authors are as follows:

- Assoc. Prof. Edwin Yeow and Dr. Shengtao Lu provided the initial project direction and edited the manuscript drafts.
- Assoc. Prof. Edwin Yeow, Dr. Shengtao and I prepared the manuscript drafts.
- Other than AFM and ITC experiments, all experiments were carried out by Dr. Shengtao Lu and me. Data analysis was performed by Dr. Shengtao Lu and me with guidance of Assoc. Prof. Edwin Yeow.
- Dr. Wuguo Bi assisted AFM experiments and analyzing data.
- Dr Sheetal Sinha assisted in ITC experiments and analyzing data.
- Mr. Arnold Subrata assisted in synthesis of silica particles and polymers.
- Prof. Bengang Xing assisted in manuscript revision.

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Abstract

This thesis focuses on the design and development of novel antimicrobial nanoplatform to combat bacterial infections. We have designed and characterized several new nanoplatforms by integrating multiple materials such as nanoparticles, polymers, organic molecules and antibiotics to combat different types of bacteria.

Chapter 1 provides a general introduction to antibacterial therapy including two strategies, one is drug-based antibacterial strategy, the other one is nanoparticle-based antibacterial strategy. For nanoparticle-based antibacterial strategy, nanoparticles serving as vehicles for drug delivery and nanoparticles with intrinsic antibacterial capacity such as photodynamic and photothermal ability will be introduced. And then attention is given to swarming motility of bacteria and we discuss how the swarming motion occurs and how antimicrobial resistance is induced by swarming motility.

In chapter 2, an antibacterial drug release system that is triggered into action upon sensing the motion of swarmer Gram-negative bacteria is introduced. A copolymer (pNIPAAm-co-AEMA) which displays affinity to Gram-negative bacteria, is electrostatically attached to the surface of mesoporous silica particles (drug carriers) in order to seal in the loaded drug, tobramycin. When swarmer *P. mirabilis* cells approach and come in contact with the particles, the copolymer chains bind to the motile Gram-negative cells and are stripped off the particle surface; hence releasing tobramycin into the swarmer colony and inhibiting its expansion.

In chapter 3, an efficient method of bio-conjugating Van to bacteria is proposed using near-infrared (NIR)-light activation. A Nd^{3+} -sensitized upconversion nanocrystal (UCNC) decorated with toluidine blue O (TB) on its surface undergoes upconverted energy transfer from the UCNC to TB when excited by 808 nm light. The photoexcited TB then catalyses the

conversion of the dihydrotetrazine (dHTz) moiety in a Van-dHTz conjugate system to tetrazine which undergoes efficient inverse electron demand Diels-Alder reaction with prior attached norbornene molecules on bacterial cell walls. MIC of attached Van is reduced by 6- to 7-fold as compared to neat Van.

In chapter 4, an improved photothermal antimicrobial method by conjugating photothermal agents onto bacterial cell wall is designed. Following the conjugating procedure described in chapter 3, ATTO740 is attached onto bacterial cell walls by covalent bonding. This attachment is controllable by switching on/off a 808 nm light source. The enhanced affinity of ATTO740 to bacteria by covalent bonding improves photothermal activity. Bacterial mortality increases ~20% as compared to unattached ATTO740.

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Chapter 1 General Introduction

1.1 Antibacterial therapy strategies

Antibiotic resistant bacteria strains have posed to be a major challenge in public healthcare due to the higher medical costs, longer hospitalization and increased mortality of patients. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain that has adapted to develop resistance towards penicillin and cephalosporins, typical generation one drugs used in combating infections. Patients infected with MRSA will not be able to respond to these typical generation one drugs used to combat infections and thus, highly susceptible to a myriad of diseases. In 1980s, Vancomycin was prescribed to combat MRSA as it stimulates the apoptosis of bacteria through an alternate mechanism, the inhibition of bacteria cell wall synthesis. However, the imprudent usage of antibiotics has accelerated antibiotic resistance to vancomycin, giving rise to vancomycin resistant *Staphylococcus aureus* (VRSA), first reported in 1986 in Europe and 1987 in the United States¹. With no new classes of antibiotics discovered since 1980s, the rapid rate at which antibiotic resistance occurs far exceeds the average time taken for the development of new antibiotics, escalating the threat of drug-resistance infections. Figure 1.1A illustrates that organisms possessed their own innate immune mechanism against bacterial infections including macrophage and neutrophils' recruitment and activation, antimicrobial activities of specific antibodies, generation of reactive oxygen species (ROS) and antimicrobial activities of Fc receptors

and complement receptors². In addition, the drug resistance evolved by bacteria has emerged due to lipopolysaccharide (LPS) mutant, porin mutant or loss, overexpression of efflux pumps, antibiotic target modifications and hydrolysis by β -lactamase (Figure 1.1B)².

A viable way to eliminate multi-drug resistant bacteria is to find alternative methods instead of using new drugs. In this aspect, nanomaterials have shown great advantages over traditional antibiotics owing to their physical and biochemical properties. For example, chemical modification on the nanoparticle surface can provide effective antibacterial target site³. In addition, the large surface area of the nanoparticle enables close interactions between nano antibacterial agent and bacterial membrane. Figure 1.1C shows the probable antibacterial mechanism evolved by nanomaterials, such as the generation of ROS, the disruption of cell membrane and damage to proton efflux pumps. Some metals, metal oxides and metal halides in the form of nanoparticles have also been utilized for the treatment of biofilms and antibacterial therapy⁴. Among these nanoparticles, Au nanoparticles show a promising therapeutic intervention for bacterial infections as they are considered safe, cost-effective and eco-friendly. Au nanoparticles are able to undergo a myriad of processes to stimulate the apoptosis of bacteria cells: impairment of cell wall synthesis, prohibition of DNA replication and damaging lipids and proteins essential for its survival.⁴ On the other hand, Ag nanoparticles, another representative antimicrobial nanomaterial, release Ag^+ ions which interact critically with the DNA of microorganisms to inhibit DNA replication and cell division⁵.

Figure 1.1 A) Bacterial innate immune mechanisms. B) Several common drug resistance mechanisms induced by bacteria. C) Probable antibacterial mechanism developed by nanomaterials². This figure is adapted from reference 2.

1.1.1 Drug-based antibacterial approach

Traditional antibacterial agents mainly focus on antibiotics which is drug molecules. A common used distinction among antibiotics is bactericidal and bacteriostatic antibiotics⁶. Bactericidal drugs directly cause death and disruption of bacterial cell wall

or bacterial cell membrane or bacterial DNA. For example, β -lactams primarily acts on bacterial cell wall, daptomycin destroys bacterial cell membrane and fluoroquinolones damages bacterial DNA. Bacteriostatic drugs inhibit bacterial replication without killing the organisms. For example, most bacteriostatic drugs like sulfonamides, tetracyclines and macrolides act by inhibiting protein synthesis.

To improve the antibacterial efficiency of antibiotics, some scientists use antimicrobial combinations to treat some bacterial infection⁶. Although a single-agent therapy is generally preferred, a combination of two or more antibiotics is recommended in some special cases. For example, in the treatment of endocarditis caused by *Enterococcus* with a combination of penicillin and gentamicin, the addition of gentamicin to penicillin has been shown to be bactericidal, whereas penicillin alone is only bacteriostatic, and gentamicin alone has no significant activity. Antimicrobial combination is a good way to shorten curative time and enhance antimicrobial efficiency.

Another promising method is to bioconjugate antibiotics onto bacterial cell wall. Some groups have reported drug molecules attached onto bacteria can enhance binding affinity between drugs and bacteria to increase drugs' antibacterial activity. Some drug dimers or drug derivatives attached onto bacterial targeting ligands have a dual or multiple antibacterial effect.

1.1.2 Nanoparticle-based antibacterial approach

Nanoparticle-based antibacterial strategy mainly includes two main approaches: 1)

nanoparticles serving as vehicles for drug delivery systems; 2) nanoparticles with intrinsic antibacterial activity. For the drug delivery systems, many kinds of delivery systems with different triggered motivations have been investigated, such as pH-triggered drug delivery system, light-triggered drug delivery system and mechanical force-triggered drug delivery system. For the nanoparticles with intrinsic antibacterial activity, there are two critical antimicrobial therapies based on nanoparticles' intrinsic activity, antibacterial photodynamic therapy and antibacterial photothermal therapy.

1.1.2.1 Nanoparticles serving as vehicles for drug delivery systems

Nanomaterials as antimicrobial delivery vehicles have become an attractive tool for enhancement of antibacterial efficacy as these nanoplateforms can improve the solubility of hydrophobic antibiotics, increase drug retention time, sustain stable drug release and specifically target at the site of infection for drug delivery⁷. An optimal design of nanomaterial-based drug delivery system should involve several features as follows: 1) minimum toxicity toward healthy cells; 2) increased antimicrobial efficiency; and 3) maximum amounts of loaded drug concentration toward infection sites⁸. The design of stimuli-response drug delivery systems is highly attractive as they can anchor onto malignant cells and react in a dynamic way according to different micro-environmental factors. In the following, pH-triggered drug delivery systems and light-triggered drug delivery systems, two representative delivery systems based on exogenous and endogenous stimulus, will be discussed.

The pH of bacterial infections is acidic, generally $\text{pH} \leq 5$, and this acid micro-environment can be exploited to design pH-triggered drug delivery nanoplateforms. Ninan et al. fabricated a pH-controlled hydrogel scaffold combining carboxylated agarose/tannic acid with zinc ions⁹. Tannic acid is a weakly acidic polyphenolic compound which is used as an antibacterial agent. In this drug delivery nanoplateform, tannic acid maintained a sustainable release at acidic pH at the site of bacterial infection and exhibit negligible release at neutral or alkaline pH found in healthy tissues (Figure 1.2). Kalhapure and his colleagues synthesized chitosan nanoparticle loaded with vancomycin modified with anionic gemini surfactant (AGS) which rendered chitosan nanoparticles pH-responsible¹⁰. This vancomycin delivery system showed a higher antibacterial efficacy at acid pH than at neutral pH against *S. aureus* and MRSA.

Figure 1.2 Scheme of the fabrication of pH-controlled hydrogel scaffold. This figure is adapted from reference 9.

For light-triggered drug delivery systems, photodynamic antimicrobial chemotherapy (PACT) is a representative treatment. Its antimicrobial mechanism is based on the visible light, photosensitizers and oxygen which can generate ROS such as superoxide anion $\cdot\text{O}_2^-$, peroxide $\cdot\text{O}_2^{2-}$, hydrogen peroxide H_2O_2 , hydroxyl radicals $\cdot\text{OH}$, and hydroxyl OH^- ions that interact with the thiol groups in the protein of the bacterial cytoplasm and cell membrane causing damage to bacterial growth and reproduction¹¹. The bacterial resistance against PACT does not occur even with high concentrations of photosensitizers, reactive oxygen species, and visible light. Thus, the commonly generated resistance problem is avoided by this approach. However, photodynamic antimicrobial chemotherapy has several limitations for in vivo therapy

currently due to the following issues. First, commonly used photosensitizers typically absorb UV-visible light (400-700 nm), which restricts the penetration depth of light into the human skin. This could be circumvented if a longer wavelength of light (> 700 nm) is used to increase the penetration depth. Secondly, the dissociative photosensitizer molecules in organism tissue are accessible to metabolize into in vitro and lack selectivity towards target cells, which decreases the efficacy of photodynamic antimicrobial chemotherapy. Thirdly, typically used photosensitizers are organic dye molecules which have poor solubility in water, making them incompatible for usage in biological tissue.

Some solutions are provided for the aforementioned problems. First, it is widely established that when the wavelength of light is longer, the penetration depth of light into skin is deeper. Therefore, near infrared (NIR) photosensitizers were developed to be activated upon NIR excitation which penetrates deeper into skin. Second, nanomaterials can be used as nano-carriers to deliver the photosensitizer to target sites making it possible to deliver hydrophilic dyes to enable efficient, low toxicity and selective therapy. Third, an antimicrobial therapy platform based on nano-carriers and NIR photosensitizers can be constructed to significantly enhance the efficiency of photodynamic antimicrobial chemotherapy. Upconversion nanoparticles (UCNPs) can be excited by lower energy radiation (NIR light) to result in high energy emission (UV and visible light) through the absorption of two or more photons, which is ascribed to an anti-Stokes mechanism. UCNPs have several advantages that are critical in the field of biological therapy. First, UCNPs can emit UV and visible light upon NIR irradiation

(980 nm or 808 nm) with large Stokes shift up to 300 nm which decrease the disturbances from light source. Secondly, biological tissue scarcely absorbs NIR light, thus the signal noises from organisms will be greatly reduced. Third, the NIR light can penetrate deeper into biological skin than the commonly used UV and visible light. Narrow emission peak, good photostability, low toxicity and low photobleaching are the advantages of using UCNPs.

1.1.2.2 Nanoparticles with intrinsic antibacterial activity

Antibacterial photodynamic therapy

Antibacterial photodynamic therapy has been a promising strategy to treat bacterial infection recently. Some nanoparticles can work as photosensitizer which can generate reactive oxygen species (ROS) after light activation. ROS mainly including superoxide anion $\cdot\text{O}_2^-$, hydrogen peroxide H_2O_2 , peroxide $\cdot\text{O}_2^{-2}$, hydroxyl OH^- and hydroxyl radicals $\cdot\text{OH}$, react with thiol groups in proteins, enzymes, DNA and bacterial cell membrane to inhibit natural bacterial growth and reproduction¹². Silver nanoparticles and titanium dioxide nanoparticles are two well-known inorganic materials acting as efficient antimicrobial agents.

Silver nanoparticles can damage the bacterial cell membrane by accumulating on the surface of cell membrane, destroying the permeability of cell membrane, and finally causing the leakage of cytoplasm component. It is reported that silver ion can inhibit the protein biosynthesis and disrupt the structure of essential proteins by binding to thiol and amino groups found in amino acid due to silver ion's strong affinity for sulfur

and nitrogen¹³. Interestingly, the size is an essential factor in the bactericidal properties of these nanoparticles. Silver nanoparticles with a smaller size exhibits a stronger antibacterial effect than those with a larger size because the smaller silver nanoparticles can accumulate on the cell membrane, thus promoting more damaging interactions. Nevertheless, small silver nanoparticles can easily aggregate into large nanoparticles to lose the antimicrobial activity. Apart from size, the shape and surface coating can also affect the antibacterial treatment of silver nanoparticles. Figure 1.3 shows three most common mechanisms of Ag nanoparticles toxicity, 1) disruption of DNA replication and ATP biosynthesis by silver ions; 2) damage to cell membrane; and 3) generation of ROS.

Figure 1.3 Interactions between nano-scaled silver and bacteria¹⁴. This figure is adapted from reference 14.

Silver ions released by silver nanoparticles interact with the negatively charged lipopolysaccharide found on bacteria cell membrane. This causes an alteration of the electrochemical potential between the intracellular and extracellular membranes, resulting in cell lysis⁵. The key factor in administering effective and efficient therapy is the availability of oxygen, and delivery of these therapeutic nanoparticles to the infection site. ROS delivery agents such as ROS gel and ROS nanomaterials are commonly used to ensure the sustainable, slow release towards the infection site. Antimicrobial metallic nanoparticles can be embedded in biopolymers to form nanocomposites with enhanced biocompatibility and stability to control the release of antimicrobial metallic nanoparticles¹⁵. Some ligands such as polyethyleneimines, chitosan and glucosamine modified on nanoparticles can strengthen their uptake into the bacterial cells and improve therapeutic efficacy.

Titanium dioxide nanoparticles (TiO₂ NPs) stimulates bacteria apoptosis via photodynamic activity. TiO₂ NPs generates ROS upon excitation with ultra-violet light. These ROS disrupts amino acid biosynthesis and DNA replication in bacterial cells. The process of ROS generation and the antimicrobial mechanism of TiO₂ NPs are illustrated in Figure 1.4A and B, respectively¹⁶. Roy et al. synthesized nano sized TiO₂ by using citric acid and dextrose¹⁷. The antibacterial activities of TiO₂ NPs were evaluated by using different concentrations against *Methicillin-resistant Staphylococcus aureus* (MRSA), which showed that TiO₂ NPs have an enhanced effect on antibacterial activities compared to traditional antibiotics. Versatile drug delivery systems were fabricated by doping TiO₂ NPs as a photosensitizer.

Figure 1.4 A) ROS generation mechanism of TiO₂ nanoparticles. B) Antibacterial mechanisms of metal nanoparticles (e.g., Ag NPs, TiO₂ NPs and ZnO NPs)¹⁶. This figure is adapted from reference 16.

Antibacterial photothermal therapy

Photothermal therapy (PTT) has attracted researchers' attention due to its unique features, for instance photothermal agents (PTAs) which are the most essential element in PTT can transform light into heat, causing cell death. Bacterial cells can be effectively killed when the cell temperature is above 42 °C since high temperature causes protein inactivation, membrane rupture and permanent cell damage¹⁸. Moreover, PTT can elevate temperature around the site of bacterial cells and kill them while the temperature of healthy cells are kept normal to avoid side effects. PTT has also been

developed in the treatment of bacterial infection ¹⁹. Near-infrared (NIR) light has been extensively utilized as light source due to its deeper penetration and lower absorption by water. The frequently used photothermal agents generally absorb within a band gap of NIR light. Ideal photothermal agents should have strong absorbance in the NIR region and convert the absorbed NIR light energy into heat²⁰. Furthermore, an ideal photothermal agent also has a high photothermal efficiency, good biocompatibility and are easy to synthesize and modify²¹. Four typical types of photothermal agents have been developed ²² as shown in Figure 1.5, i) noble metal-based nanoparticles; ii) carbon-based nanomaterials; iii) semiconductors-based nanomaterials; iv) and organic molecules-based nanomaterials. Herein, we will mainly focus on noble metal nanoparticles and carbon nanomaterials.

Figure 1.5 Typical classifications of photothermal nanomaterials. This figure is adapted from reference 22.

Noble metal-based nanomaterials

The representative noble metal nanomaterials as photothermal agents are Au and Ag nanoparticles, supplying a versatile platform for biological applications in the field of tumor diagnosis and therapies. To understand the working principles of noble metal nanomaterials such as Au and Ag nanoparticles for photothermal therapy, localized surface plasmon resonance (LSPR) must first be introduced. LSPR refers to the collective oscillation of electrons on the surface of metal structure. After absorbing NIR light, a resonance effect due to the interaction between the conduction electrons of metal nanoparticles with incident photons occurs. This resonance effect can generate a thermal effect and increase the temperature rapidly²³. Moreover, the LSPR band and photothermal efficiency can be tuned by changing the size, morphology and doped elements of the noble metal nanoparticles²⁴.

Au nanomaterials with various shapes and sizes, such as nanospheres, nanorods, nanoplatelets, nanotriangles and nanoprisms from 1 nm to 1 μ m, have been synthesized in recent years. Jiang et al. reported that the photothermal efficiency can be tunable with variable sizes of gold nanoparticles²⁵. Gold nanoparticle with sizes ranging from 5 to 50 nm were synthesized by using reduction agents like sodium borohydride and sodium citrate. The light-to-heat transfer efficiency decreases from 0.803 to 0.650 as the size of gold nanoparticles increases from 4.98 to 50.09 nm, respectively. Singh et al. reported the synthesis of gold coated-liposomes nanoparticles with loaded curcumin²⁶. Curcumin loaded liposome nanoparticles (Lipos Cur NPs) were first prepared by thin film hydration method and gold was coated on Lipos Cur NPs to form Au coated-

liposomes nanoparticles with loaded curcumin (Au-Lipos Cur NPs). The gold coating absorbs NIR light by LSPR to generate heat which destabilizes the liposome nanoparticles causing the release of curcumin. The photothermal efficacy from gold coating would induce irreversible cellular damage and the release of curcumin would also enhance the cancer cell cytotoxicity. This “on demand” curcumin delivery system using the NIR light can have a dual effect from gold photothermal therapy and curcumin itself adjuvant therapy. Some modifications on Au nanoparticles were developed like synthetic peptide and polymer modifications. Rovati et al. reported that Au nanostar monolayers were grafted on a coverslip and the hydrophilicity or hydrophobicity is optimized by coating with the proper thiol²⁷. High hydrophilicity or hydrophobicity can suppress the biofilm formation and bacterial colonization. This gold nanostar monolayers modified with thiol functional group can kill 99.99% of *S. aureus* and *E. coil* under the irradiation of NIR light (808 nm, 0.264W cm⁻²) for 30 min.

Ag nanoparticles have been extensively studied due to its unique electrical, physical, and biological properties²⁸. Ag nanoparticles work not only as a photothermal agent due to its LSPR performance but also as an antimicrobial agent due to its special antibacterial properties²⁸. Ag ions can be transported into cells, inhibit the DNA replication by binding to DNA, and then restrain cell division. Ag ions can also destroy the respiratory system of cells by generating reactive oxygen species (ROS) to cause cell death. Wang et al. developed a versatile PTT system targeting liver cancer cells including Janus mesoporous silica nanoparticles (JNPs), Ag nanoparticles, folic acid (FA) and indocyanine green (ICG) which were combined together to form a FA-

targeting, ICG loaded JNPs (FA-JNPs@ICG) that overcomes the poor photostability and aqueous stability of ICG²⁹. The ICG-based photothermal effect can accelerate the release of ICG and Ag ions, the former was for killing the tumor cells through heat generation, and the latter acting as an antimicrobial agent to generate ROS to cause cell necrosis. It was shown that the surrounding temperature of FA-JNPs@ICG can rise to 51.6 °C upon the irradiation of NIR light (808 nm, 1 W cm⁻²). Unfortunately, the aqueous aggregation of Ag nanomaterials has restricted their further utilization. The modification on Ag nanomaterials was able to improve its solubility and homogeneity. For instance, Li et al. directly synthesized Ag nanoclusters on reduced graphene oxide (AgNC@rGO) by reduction from glutathione (GSH). The aqueous aggregation of Ag nanoclusters was obviously circumvented due to the role of reduced graphene oxide.

Due to the LSPR effect of Au and Ag nanomaterials especially for the antimicrobial effect of silver ions, some research which combine Au and Ag nanomaterials were reposted in recent years. Chen et al. synthesized a bimetallic Au-Ag nanoparticles by using a sodium citrate thermal reduction method³⁰. The experimental results showed that the photothermal efficiency of bimetallic Au-Ag nanoparticles was 41.37% which is much higher than the blended Au-Ag nanoparticles (31.41%). Some research groups focus on the fabrication of upconverting nanoplateforms. Qu's group has presented an innovative idea by constructing a multifunctional core-shell nanocomposite (NaYF₄: Mn/Yb/Er@MB with decorated CuS nanoparticles) to exhibit a superior antibacterial photothermal effect towards drug-resistant bacteria (Figure 1.6)³¹.

Figure 1.6 A) The synthesis process of NaYF₄: Mn/Yb/Er@MB@CuS and the characterizations of NaYF₄: Mn/Yb/Er@MB@CuS. B) the antibacterial photothermal activities of NaYF₄: Mn/Yb/Er@MB@CuS. This figure is adapted from 31.

Carbon-based nanomaterials

The carbon-based nanomaterials such as carbon quantum dots (CQDs), graphene-based nanomaterials (GBNs) and carbon nanotubes (CNTs) have obtained considerable attention and attraction from many researchers due to large surface area, unique optical/thermal properties and tunable functional groups. The general carbon-based nanomaterials are capable of absorbing a broad band light from ultraviolet to near-infrared wavelengths³².

Carbon quantum dots are a new class of ultra-small carbon nanoparticles with good fluorescent properties. Their excellent fluorescent properties originate from broad excitation spectrum, tunable emission band and outstanding photostability, which led to the extensive applications of carbon quantum dots in the field of bioimaging. Furthermore, they have promising application in biosensing, drug delivery and electrochemical devices on account of their small size, tunable photoluminescence,

good biocompatibility, chemical inertness and low toxicity³³⁻³⁴. Bao et al. developed nascent CQDs with a controllable optical bandgap from urea to citric acid ligands using solvothermal method³⁵. The as-prepared CQDs showed a strong absorption in the region from red to NIR wavelengths, and the maximum absorption coefficient and the maximum emission coefficient peaked at 600 nm and 720 nm, respectively. The light-to-heat transfer efficiency can reach 59.19% under irradiation of red light (655 nm, 1 W cm⁻²) for 10 min. Geng et al. synthesized nitrogen and oxygen doped CQDs (N-O-CDs) with promising absorbance in the NIR region *via* one step molecular fusion route³⁶. This method utilized a low power laser (0.8 W cm⁻²) to avoid side effects from high light intensity, while achieving 100% tumor cells destruction with 38.3% photothermal efficiency. Zheng et al. discovered a novel method to fabricate CQDs as a scaffold for therapeutic, diagnostic and targeting agents³⁷. In this work, they used a hydrophobic cyanine dye (CyOH) to fabricate NIR fluorescent carbon dots (CyCD) to overcome the poor hydrophilicity of CyOH. CyCD showed outstanding hydrophilicity and excellent photostability to improve the utility of CyOH as tumor imaging agent and PTT agent. The experimental results showed CyCD has potential in tumor imaging and therapy.

Graphene-based nanomaterial is a two-dimensional carbon crystal which have attracted vast interests due to its large surface area, high electron motility and excellent thermal conductivity³⁸⁻³⁹. Reduced graphene oxide (rGO) has been exploited as photothermal agents⁴⁰⁻⁴¹. For example, Matteini et al. fabricated a platform which can remotely control the NIR laser to trigger drug release from graphene⁴². Here graphene

not only plays a role of accommodating drug payloads but also converts light to heat.

Carbon nanotubes were first discovered in 1993 by Iijima et al, and are also referred to as single-wall carbon nanotube (SWCNTs) or multi-wall carbon nanotube (MWCNTs), consisting of sp^2 hybridized carbon atoms, by rolling up a single sheet graphene or multiple sheets graphene⁴³. Owing to the effective absorbance of carbon nanotubes in NIR region and good capacity to transfer electronic excitations into molecular vibrations to generate heat, CNTs have become a promising photothermal agent in tumor targeting and therapy⁴⁴⁻⁴⁷. Kim and his colleagues used self-assembling CNTs to bind onto bacterial membrane and transform light to heat to implement 100% bacteria destruction⁴⁸.

1.2 Swarming motility of bacteria

There are five different bacterial motions including swimming, gliding, twitching, sliding and swarming⁴⁹⁻⁵². Swarming motility is a rapid multicellular movement across solid or semi-solid surface by the flagella rotation, unlike the other four types of bacterial translocations which typically occur in individual planktonic bacterial cells in a liquid environment⁵². The speed of some swarming bacteria can reach several micrometers per second⁵³. Figure 1.7 shows macroscopic migration of wild-type *E. coli* swarmer cells on the surface of agar plate at different time. Swarmer cells are in a monolayer and keeping immobile at the beginning edge of swarming colony. Swarmer cells located in multilayer move faster than those in a monolayer⁵⁴.

Figure 1.7 Macroscopic migration of wild-type *E. coli* swarming colony on a 0.45% (w/v) Eiken agar at different time (e.g., 0, 60, 120 and 180 sec). This figure is adapted from reference 54.

1.2.1 Requirements for swarming motility

Flagellar biosynthesis, water and surfactant, and cell-cell interactions are three necessary conditions for swarming motility⁵².

Flagella biosynthesis

Flagella is a filamentous protein structure on the peripheral of a bacterium consisting of a helical filament, basal body and a hook⁵⁵. The helical filament is a left-handed polymer capped flagellin proteins with a ~ 15 mm length and 12-25 nm diameter outside the bacterial cell⁵⁶⁻⁵⁸. The basal body consisting of four rings and a rod is embedded into the bacterial cell wall⁵⁹. The hook connects the helical filament and basal body in

the middle (Figure 1.8). Flagella decorated with fluorescent dyes can be observed by fluorescence microscope⁶⁰. The numbers of flagella on the peripheral of a bacterium will increase for swarmer phenotypes⁶¹. For example, the number of peritrichous flagella of *S. Typhimurium* and *E. coli* becomes double from initially around 10⁶². Flagella is the driving force for rapid and multicellular movement across a surface. It was reported that several flagella may become bundled together and rotate when bacterial cells moved side by side, thus effectively increasing the stiffness of flagella and its moving force⁶³. The role of flagella during swarming behavior was demonstrated by acquiring mutations that enhance or weaken the expression of flagella genes⁶⁴⁻⁷⁰. The results showed that mutation with enhanced flagella genes expression possessed increased number of flagella, and mutation with weaken flagella genes expression had a reduced number of flagella and less active swarming motions⁷⁰.

Figure 1.8 A cartoon figure showing components of flagella of *Salmonella enterica* serovar Typhimurium⁷¹. This figure is adapted from reference 71.

Water and surfactant

Water is a crucial factor in swarming motility^{49, 53}. Bacterial cells are unable to swarm on a completely dry surface. The most widely used semi-solid medium for swarming is agar. The experimental results showed that only 0.45-1% agar (w/v) can support bacterial swarming motions⁷². Once the concentration of agar exceeds 2%, most bacterial cells will not swarm and instead grow into planktonic colonies⁷³. However, agar plate with low agar concentrations are also not suitable for swarming movements.

The gel pore size on the concentration of agar (< 0.4%) plate is larger than the radius of bacterium which allows bacterial cells to go into the networks of the agar polymers and swim⁷⁴. Swarming motility requires the production of a viscous slime layer, which is defined as surfactant⁷⁵. The surfactant, essential for the formation of swarming motility, is generally secreted by bacteria to reduce the surface tension between the bacterial cell and semi-solid surface⁵². Surfactant needs to be secreted at a high concentration to enable the swarming mobility of the bacteria⁵⁴. The commonly known surfactants include polysaccharide, lipopolysaccharide (LPS), lipopeptide and glycolipid⁵⁴. Lipopolysaccharide is the most dominant component when *P. mirabilis* are swarming⁷⁶. However, data have shown that LPS-deficient mutants cannot swarm⁷⁶. The mutant of *S. typhimurium*, where a *waaG* gene which partakes in the biosynthesis of LPS to generate extensive amounts of wetting matrix are overexpressed, possessed an enhanced drastic swarming motility⁷⁷. Figure 1.9 shows the chemical structures of several surfactants, and swarming colonies and surfactant-defective mutant non-swarming colonies⁵⁴.

Figure 1.9 A) Chemical structure of colony migration factor and the swarming colonies of wild-type *P. mirabilis* and Cmf-defective mutant. B) Chemical structure of serrawettin W2 and the swarming colonies of a *S. liquefaciens* mutant with 40 and 0 $\mu\text{g mL}^{-1}$ serrawettin on agar plate. C) Chemical structure of surfactin and swarming colonies of wild-type *B. subtilis* and surfactin-defective mutant. D) Chemical structure of mono-rhamnolipid and swarming colonies of wild-type *P. aeruginosa* and RL-defective mutant. This figure is adapted from reference 54.

Cell-cell interaction

Cell-cell interaction is critical for swarming motility and is allowed for physical interactions between bacterial cells. Swarmer cells align parallel to each other and coordinate their movements, swaying side by side like rafts. The flagella of adjacent bacteria cells bundle together during the surface swarming migration. Transmission electron microscope (TEM) was used to visualize the slime and bundled flagella of

swarmer cells by Stahl et al in 1983⁶³. Scanning electron microscope (SEM) results shows swarmer cells are aligned to form multicellular rafts and appear to be interwoven to form helical connections in Figure 1.10⁷⁸.

Figure 1.10 SEM of swarming fronts of wild-type *P. mirabilis* and transposon mutants exhibiting helical connections⁷⁸. Bars = 1 mm. This figure is adapted from reference 78.

1.2.2 Swarming-associated phenotypes

A swarming lag, swarmer cell elongation and the formation of colonies with different shapes are three phenotype features during swarming.

Figure 1.11 A cartoon depicting transformation process from planktonic cells to swarming cells. This figure is adapted from reference 52.

A swarming lag is needed to transit from individual swimming to multicellular swarming⁷⁹. Swimmer cells must go through a change to become swarmer phenotypes. Two features will be achieved after a swarming lag time: high density of cell population and hyperflagellation⁶⁸. The swarmer cells are longer than the normal planktonic swimmer cells. *P. mirabilis* cells are 10-80 mm long when they become swarming, which is 10 to 50 times in length than planktonic cells⁵⁴. Moreover, the increased number of flagella also elongate the swarmer cells. The transformation process from planktonic cells to swarming cells is demonstrated in Figure 1.11. Compared to a relatively small circular colony formed by planktonic cells, swarming bacteria form macroscopic colonies with different morphologies⁵². Several examples are listed in Figure 1.12, the *Bacillus subtilis* (ATCC 3610) swarmer cells spread throughout the whole petri dish to form a featureless expansion colony⁵². A bull's eye colony pattern

was formed by *Proteus mirabilis* (ATCC 7002)⁵². In addition, some shapes like dendrites and vortex can be formed by the swarmer cells of *Pseudomonas aeruginosa* and *Paenibacillus vortex*.⁵²

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Figure 1.12 Various swarming colonies formed by different bacteria. This figure is adapted from reference 52.

1.2.3 Swarming induced antimicrobial resistance

It has been reported that swarming bacterial colony exhibited an enhanced resistance against multiple antibiotics compared to swimming cells of the same strain. Kim and Surette reported that swarmer cells of *Salmonella enterica* showed elevated resistance to a wide range of antimicrobial agents⁸⁰. E-test strip results showed swarmer cells displaying elevated resistance to a variety of antibiotics including those targeting cell membrane, DNA replication and protein biosynthesis⁸¹. Lai et al. investigated how swarming behaviors were coupled to widespread antibacterial resistance⁸². Swarming cells of *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli* showed higher resistance than planktonic cells against 13 classes of antibiotics.

Unlike the planktonic cells, minimum inhibition concentration (MIC) is not easily determined by using various concentrations of drugs as swarmer cells easily move across a semi-solid surface. A straightforward method to determine the MIC value for swarmer cells is the agar diffusion method. The agar diffusion method is conducted as follows. First, an agar plate is spread with bacteria. Second, a small paper disc soaked with a gradient concentration of antibiotics is placed onto the agar plate. Third, swarming bacteria encounters the antibiotics, and the bacteria will be killed. Thus, forming a circular zone without bacteria called inhibition zones. By comparing the size of inhibition zone, the ability of antibiotics to inhibit bacterial growth is evaluated. The larger the inhibition zone is, the antibacterial capacity of antibiotics is stronger. Conversely, if the bacteria are insensitive or resistant to the antibiotic, the inhibition zone will not appear. Based on the agar diffusion method, a more efficient method, the

E-test strip has been developed. The E-test strip is soaked with a gradient concentration of drugs from the top to bottom and is placed on the agar plate. Different positions of the strip contain different concentrations of drugs, thus the inhibition zones at different positions are of different sizes. The MIC value can then be read directly from the scale of the E-test strip at the edge of relatively small inhibition zones.

1.2.4 Mechanism of swarming induced antimicrobial resistance

Several antibacterial resistance mechanisms have been investigated mainly involving enzymatic drug inactivation and prevention of cellular uptake. β -lactam is used to demonstrate the drug resistance mechanism of enzymatic drug inactivation here. Bacterial resistance genes can upregulate some targeting enzymatic activities. Bacteria with drug resistance can enzymatically hydrolyze the β -lactam bond, which has the most predominant function within β -lactam antibacterial activities. The outer lipid composition and porin channel activity of drug resistant bacteria will be transformed to prevent the cellular uptake of antibiotics. According to recent research reports, it is widely believed that swarming induced drug resistance is derived from high cell density and swarming motion dynamics of swarmer cells⁸³. In addition to high cell density and swarming motion dynamics, some genes are upregulated or downregulated during swarming movements.

A study that investigated how cell intensity influences drug resistance of swarmer cells was carried out by Butler et al⁸³. Within this research work, two reasons for high drug resistance of swarmer cells caused by high cell density were discussed: 1) faster

migration speed allowed less casualties compared to swarmer cells at a slow migration speed due to the less exposure time to the lethal antibiotic; 2) multi-layer swarmer cells at the swarming front can tolerate more violent antibacterial activity as swarmer cells at the bottom layer can sacrifice themselves to protect swarmer cells at the top layer in a safe environment. Thus, a higher MIC value can be observed from E-test strip.

Some work has been exploited to achieve higher antibacterial efficacy by disturbing the swarming motility which illustrated that swarming motion dynamics is relevant to enhancement of antibacterial resistance⁸¹. Moreover, some genes can be overexpressed during swarming to resist with drugs at a higher concentration. Kim et al. found that swarmer cells of *Salmonella enterica*, whose LPS related genes were upregulated and then resulted in a more charge-positive LPS, showed less affinity to cationic antibiotics polymyxin, which triggered a higher resistance of *Salmonella enterica* against cationic antibiotics⁸¹.

1.3 Aims

Several novel antimicrobial nanoplateforms are developed in this thesis to combat bacterial infections by integrating multiple materials such as nanoparticles, polymers, organic molecules and antibiotics. Mechanical force and light activation are the primary means used for antibacterial therapy in this thesis.

In chapter 2, we fabricated an intelligent drug release system (IDRS) based on Motion-Induced Mechanical Stripping (MIMS). This drug release system is triggered by mechanical force, which provides a new method among different triggering methods

for drug release systems.

In chapters 3 and 4, an efficient method of bio-conjugating antibiotic/photothermal agent to bacteria is proposed using near-infrared (NIR)-light activation. Light activation is thus used to attach antibiotic/photothermal agent onto bacterial cell wall. This method provides a controllable method to link antibiotic/photothermal agent onto bacterial cell, which increases drugs or photothermal molecules' antibacterial efficacy. Vancomycin is used as antibiotic in Chapter 3 and ATTO740 is used as the photothermal agent in Chapter 4.

Chapter 2 Lipopolysaccharide-affinity Copolymer Senses the Rapid Motility of Swarmer Bacteria to Trigger Antimicrobial Drug Release[†]

Abstract

An intelligent drug release system that is triggered into action upon sensing the motion of swarmer *P. mirabilis* is introduced. The rational design of the drug release system focuses on a pNIPAAm-co-pAEMA copolymer that prevents drug leakage in a tobramycin-loaded mesoporous silica particle by covering its surface *via* electrostatic attraction. The copolymer chains are also conjugated to peptide ligands YVLWKRKRKFCFI-NH₂ that display affinity to Gram-negative bacteria. When swarmer *P. mirabilis* cells approach and come in contact with the particle, the copolymer-YVLWKRKRKFCFI-NH₂ binds to the lipopolysaccharides on the outer membrane of motile *P. mirabilis* and are stripped off the particle surface when the cells move away; hence releasing tobramycin into the swarmer colony and inhibiting its expansion. The release mechanism is termed Motion-Induced Mechanical Stripping (MIMS). For swarmer *B. subtilis*, the removal of copolymers from particle surfaces *via* MIMS is not apparent due to poor adherence between bacteria and copolymer-YVLWKRKRKFCFI-NH₂ system.

[†] This chapter has been published in Lu, S.; Bi, W.; Du, Q.; Sinha, S.; Wu, X.; Subrata, A.; Bhattacharjya, S.; Xing, B.; Yeow, E. K. L., Lipopolysaccharide-affinity copolymer senses the rapid motility of swarmer bacteria to trigger antimicrobial drug release. *Nature Communications* **2018**, 9 (1), 4277.

2.1 Introduction

Proteus mirabilis is a Gram-negative bacteria associated with urinary tract infections (UTIs) that often affect patients inserted with indwelling urethral catheters⁸⁴⁻⁸⁷. A unique characteristics of *P. mirabilis* is its ability to undergo cell differentiation into elongated and hyperflagellated swarmer cells that move rapidly across surfaces of solid media, including urethral catheter surfaces, in multicellular rafts^{52, 84-87}. The collective motion is believed to play a role in the initiation of catheter-associated UTIs (CAUTIs) by facilitating the dissemination of bacteria from infection sites on the catheter to the bladder^{78, 86-88}. The most common treatment of CAUTIs involves either regular replacements of infected catheters with new ones, administration of strong antibiotics or prevention of biofilm formation/encrustation, often with conflicting results^{7, 86-87}. Unfortunately, existing methods to combat CAUTIs are unable to control the release of antibiotics nor do they specifically target the initial migration of swarmer *P. mirabilis* from catheter surface to urinary tract.

The controlled release of drug is an important process as it allows the therapeutic agent to display its active form only at the infection site⁸⁹⁻⁹¹. Previous technologies on

'on-demand' release of antimicrobial drugs encapsulated within carriers (*e.g.*, particles, polymers, etc.) have utilized toxin/lipase secreted by bacteria to react with toxin/lipase-responsive materials on carriers to trigger drug release⁹¹⁻⁹⁵. Another common stimuli-responsive method utilizes the reduced pH environment in cariogenic dental biofilms ($\text{pH} \leq 4.5$) to activate drug release from pH-sensitive carriers⁹⁶⁻⁹⁷. So far, there have been no reports on controlled drug release technology based on the motion of motile bacteria.

Figure 2.1 Mechanism of Motion-Induced Mechanical Stripping (MIMS).

Antimicrobial agents are encapsulated within the pores of a mesoporous silica particle and prevented from leaking out by the bacteria recognizing copolymer attached onto the surface of the particle (left). When swarmer Gram-negative bacteria brush against the drug carrier, the polymeric chains bind selectively to the fast moving cells and are stripped off the surface of the particle; hence exposing the pores and allowing the payload to be released into the bacterial colony (right). Only the top hemisphere of the particle is shown.

In this chapter, as proof-of-concept, we will design and prepare an intelligent drug release system (I-DRS) that releases antimicrobial agents in the presence of motion of swarming *P. mirabilis*, and demonstrate the I-DRS efficacy against the spread of the swarmer colony. The I-DRS comprises of a mesoporous silica particle MCM-41 that contains an antimicrobial drug (Figure 2.1). In the absence of motile bacteria, the therapeutic payload is retained within the pores and prevented from leaking out *via* a polymeric shell, electrostatically bound to the particle surface, that blocks the pore entrances. The polymer used is a pNIPAAm-*co*-pAEMA copolymer, consisting of the monomers *N*-isopropylacrylamide (NIPAAm) and 2-aminoethyl methacrylate hydrochloride (AEMA), conjugated to peptide ligands with sequence YVLWKRKRKFCFI-NH₂ (**3** in Figure 2.2). The copolymer pNIPAAm-*co*-pAEMA is used because it is non-toxic to *P. mirabilis* at the concentrations used here, soluble in water and convenient to synthesize and functionalize. In particular, pNIPAAm-based copolymers and pAEMA have previously been utilized for drug delivery to various cells with little cytotoxicity effects⁹⁸⁻¹⁰⁰. The peptide YVLWKRKRKFCFI-NH₂ binds relatively strongly to lipopolysaccharides (LPS), found on the outer membrane of Gram-negative bacteria, with an association constant of $K_a = 0.130 \mu\text{M}^{-1}$ (Figure 2.3)¹⁰¹⁻¹⁰³. The binding of the peptide to LPS is comparable to the association between a YVLWKRKRKFIFI peptide, a sequence close to the one used in this study, and LPS ($K_a = 0.22 \mu\text{M}^{-1}$)¹⁰¹, and between other LPS binding peptides and LPS (~ 0.1 to $0.2 \mu\text{M}^{-1}$)¹⁰⁴⁻¹⁰⁵. In this way, when swarmer *P. mirabilis* cells approach close to the I-DRS, polymeric chains initially found on the surface of the particle will attach themselves

onto the fast moving cells because of the LPS-copolymer **3** binding and be pulled away (*i.e.*, mechanically stripped off the particle due to the strong force from rapidly moving bacteria); hence allowing the encapsulated drug to be released into the bacterial colony (Figure 2.1). We term this new bacteria-triggered drug release mechanism as Motion-Induced Mechanical Stripping (MIMS).

Figure 2.2 Synthetic steps for the preparation of copolymer **3**. From GPC and ¹H NMR results, $m \sim 77$, $n \sim 11$ and $p \sim 2$. Synthesis and characterization details are provided in the experimental section.

Figure 2.3 ITC thermogram of interactions between LPS (from *P. mirabilis*) and the YVLWKRKRKFCFI-NH₂. The association constant K_a between YVLWKRKRKFCFI-NH₂ peptide and LPS was determined by fitting the ITC data into a single set of binding sites. A K_a value of $0.130 \mu\text{M}^{-1}$ is obtained.

2.2 Experimental section

2.2.1 Materials and instruments

Cetyltrimethylammonium bromide, 2-aminoethyl methacrylate hydrochloride (AEMA), β -alanine, *N*-isopropylacrylamide (NIPAAm), ethyl-2-bromopropionate, maleic anhydride, *N,N,N',N',N'*-pentamethyldiethylenetriamine (PMDETA), tobramycin, toluidine blue, CuBr, tetraethoxysilane, lipoteichoic acid (LTA from *B. subtilis*, protein content $\leq 3\%$), lipopolysaccharide (LPS from *P. mirabilis*, protein content $\leq 3\%$, and *P. aeruginosa*), polystyrene latex beads and amine-modified polystyrene beads were purchased from Sigma-Aldrich. ATTO-647N-NHS ester was purchased from ATTO-TEC GmbH. 25% ammonia was purchased from Sinopharm Chemical Reagent Co. Ltd. *O*-Benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium

hexafluorophosphate (HBTU) and amino acids used for solid phase peptide synthesis were purchased from GL Biochem (Shanghai) Ltd. NIPAAm was recrystallized using hexane before use. Dicarboxy terminated poly(N-isopropyl acrylamide) (P9110E-NIPAM2COOH, Mn: 10,000) was purchased from Polymer Source. All other chemicals were used as received. Tobramycin E-test strips were obtained from bioMérieux.

Transmission electron microscopy (TEM) images of silica particles were recorded using JEM 1400 (JEOL). Mass spectra were measured using a LCQ Fleet mass spectrometer (Thermo Scientific). Nuclear magnetic resonance (NMR) spectroscopy was carried out on an Ultrashield 400 Plus NMR spectrometer (Bruker). Gel permeation chromatography (GPC) data was obtained using a 1260 Infinity II (Agilent Technologies) GPC instrument with PolarGelM column (Agilent Technologies). UV-Vis absorption spectroscopy was measured using a Cary-100 UV-Vis spectrometer (Varian). Zeta potential was measured using a Zetasizer NANO system (Malvern). High performance liquid chromatography (HPLC, Waters 2695 Separations Module) was used to quantify the amounts of tobramycin released from copolymer **3**-coated silica particles.

2.2.2 Synthesis of mesoporous silica MCM-41 particles¹⁰⁶

In a 250 mL flask, a mixture of cetyltrimethylammonium bromide (0.35 g) and 25 % ammonia (40 mL) were added into water (70 mL) and stirred at 80 °C for 30 min. Tetraethoxysilane (1.70 g) was then slowly added to the mixture and stirred at 80 °C for 2 h. The white precipitate was filtered and washed with water (4 times) and ethanol (4 times). The mesoporous silica particle MCM-41 product was dried overnight at 80 °C

and calcinated at 600 °C for 6 h at a heating rate of 1 °C min⁻¹.

2.2.3 Synthesis of bacterial recognizing peptide

The peptide sequence YVLWKRKRKFCFI-NH₂ was synthesized using solid phase synthesis method¹⁰³. MS (TOF) found: 1786.99; calculated: 1787.03 for C₈₈H₁₃₆N₂₃O₁₅S [YVLWKRKRKFCFI-NH₂+H]⁺.

2.2.4 Synthesis of 3-maleimidopropionic acid 4

In a 250 mL flask, β -alanine (0.89 g) and maleic anhydride (0.98 g) were mixed in dry THF (150 mL) and stirred for 2 days. The solvent was evaporated and toluene (100 mL) was used to suspend the white precipitate. The suspension was then stirred at 120 °C for 6 h and cooled to room temperature. Toluene was then removed by evaporation to leave a yellow precipitate. The product 3-maleimidopropionic acid **4** was first extracted from the yellow precipitate using DCM. The DCM solution was washed with NaHCO₃ solution (3 times) followed by water and brine. The organic phase was dried by Na₂SO₄ and evaporated to get the crude product which was purified using flash column with DCM. ¹H NMR (CDCl₃): δ (ppm) 6.72(s, 2H), 3.84 (t, 2H), 2.71 (t, 2H). ¹³C NMR(CDCl₃): δ (ppm) 176.2, 170.3, 134.3, 33.3, 32.5.

2.2.5 Synthesis of copolymer **1**¹⁰⁷

A mixture of *N*-isopropylacrylamide (1.98 g, NIPAAm), 2-aminoethyl methacrylate hydrochloride (0.41 g, AEMA), ethyl-2-bromopropionate (0.018 g), *N,N,N',N',N'*-pentamethyldiethylenetriamine (45 μ L, PMDETA), methanol (16 mL) and water (24 mL) in a 100 mL schlenk flask was degassed by cycles of freeze-pump-thaw. CuBr (0.015 g) was added while the mixture was frozen. The solution was then stirred under argon atmosphere at room temperature for overnight. The product was purified by dialysis and dried to obtain pNIPAAm-*co*-pAEMA copolymer **1**. The number average molecular weight $M_n = 10171 \text{ g mol}^{-1}$, weight average molecular weight $M_w = 21985 \text{ g mol}^{-1}$ and polydispersity PD = 2.16.

2.2.6 Synthesis of copolymer **2**

O-Benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (0.02 g, HBTU) and 3-maleimidopropionic acid **4** (0.085 g) were dissolved in dry DMF (5 mL) and stirred for 10 min before being added to a solution containing copolymer **1** (0.24 g)

in dry DMF (10 mL). The reaction mixture was stirred for 2 days at room temperature. DMF was then evaporated and the crude product was dissolved in water and purified by dialysis. The solution was lyophilized to obtain dry copolymer **2**.

2.2.7 Synthesis of copolymer 3

Peptide YVLWKRKRKFCFI-NH₂ (100 mg) was dissolved in PBS buffer (100 mL) and filtered using a 25 mm syringe filter with 0.45 µm membrane. Copolymer **2** (0.2 g) was then added into the filtered solution. The mixture was stirred for 3 days at room temperature and purified by dialysis. The final solution was lyophilized to yield copolymer **3**. The number average molecular weight $M_n = 14212 \text{ g mol}^{-1}$, weight average molecular weight $M_w = 43033 \text{ g mol}^{-1}$ and polydispersity PD = 3.63. Assuming a stoichiometric reaction, the ratio $m:n = 7.06:1$. This agrees with the ¹H NMR data of copolymer **3** in D₂O where peaks at $\delta(3.90 \text{ ppm})$ from H_a and $\delta(4.25\text{-}4.10 \text{ ppm})$ from H_b and H_c have an integration area ratio = 5.74:1. The values of m , n and p were therefore taken to be *ca.* 77, 11 and 2, respectively.

2.2.8 Fluorescent dye labeling of copolymer

Copolymer **2** (200 mg) was dissolved in 0.1 M NaHCO₃ solution (15 mL). ATTO-647N-NHS ester (1 mg) was added into the solution and stirred for 2 days at room

temperature. The mixture was then dialyzed and lyophilized to get copolymer **5**. A similar procedure as outlined in section 2.2.7 was performed on copolymer **5** to yield the dye labelled copolymer **6** (Scheme 2.1). On average, one dye molecule was conjugated to a copolymer chain.

Scheme 2.1 Synthetic steps to prepare copolymer **6**.

2.2.9 Determining the loaded amount of drug

A weighed amount of silica particles (~ 5 mg) were loaded with tobramycin by stirring an aqueous solution (~ 500 μ L) of tobramycin (400 mg mL⁻¹) with the particles for 3 days¹⁰⁸. The drug loaded particles were recovered by centrifugation, washed with DI water (0.5 mL), freeze dried and weighed again. The loading amount was obtained by the difference in mass of silica particles before and after loading with tobramycin. For our experiment, the amount of tobramycin loaded is 0.234 ± 0.006 mg of drug/1 mg

of silica particles.

2.2.10 E-test assay

P. mirabilis

An (4 %) agar plate was prepared in a 10-cm Petri dish. An exponential growth phase *P. mirabilis* culture was applied on the entire agar surface and dried for 15 min. A tobramycin E-test strip was placed on the center of the surface of the agar before incubation for 20 h at 37 °C.

To examine if copolymer **3** affects the viability of *P. mirabilis* when exposed to tobramycin, 1.4 mg mL⁻¹ copolymer **3** (200 µL) was evenly applied on the entire surface of an (4 %) agar and dried for 30 min. An exponential growth phase *P. mirabilis* culture was then applied on the entire agar surface and dried for 15 min. A tobramycin E-test strip was placed on the center of the agar followed by an incubation period of 20 h at 37 °C. The minimum inhibitory concentration (MIC) value was recorded following manufacturer instructions.

B. subtilis

An (4 %) agar plate was prepared in a 10-cm Petri dish. An exponential growth phase *B. subtilis* culture was applied on the entire agar surface and dried for 15 min. A tobramycin E-test strip was placed on the center of the surface of the agar before incubation for 20 h at 37 °C. The MIC value was recorded following manufacturer instructions.

2.2.11 Determination of the MIC of YVLWKRKRKFCFI-NH₂ against *P. mirabilis*

The MIC of the peptide YVLWKRKRKFCFI-NH₂ against *P. mirabilis* was determined. Mid logarithmic phase of an overnight culture in LB broth was prepared and OD₆₀₀ adjusted to 0.2. The bacterial medium (50 µL) was added to an equal volume (50 µL) of an aqueous solution of peptide of various concentrations (*i.e.*, 0, 64, 128, 256, 320, 640, 1280, 1600, 1920 and 3200 µg mL⁻¹). The mixture was incubated for 3 h at 37 °C, and a 100 times-diluted solution (10 µL) was applied onto an LB-agar plate. The minimum concentration of peptide that results in 90 % growth inhibition is taken to be the MIC value.

2.2.12 Isothermal titration calorimetry

The binding constants between peptide YVLWKRKRKFC-NH₂ and lipopolysaccharide (LPS) and between the peptide with lipoteichoic acid (LTA) were determined using a MicroCal ITC200 isothermal titration calorimeter. Peptide, LPS and LTA solutions were first prepared by dissolving each component in 10 mM phosphate buffer (pH 7.0). LTA (50 µM) was loaded into the sample cell, the reference cell was filled with the same buffer and the syringe was filled with 1mM peptide solution. Typically, 25 injections of 1.6 µL of peptide were made into the sample cell at 25 °C and the sample cell was stirred at 900 rpm. Raw data were obtained and analyzed using single set of binding sites in the MicroCal PEAQ-ITC analysis software. Association constant (K_a) was directly determined from the ITC profiles.

2.2.13 Dye and Drug Release Experiments

The capability of copolymer **3** to encapsulate a payload within mesoporous silica particles was demonstrated by performing UV-vis absorption spectroscopy on toluidine blue (TB) dye retained in the particles. TB was loaded into the silica particles by dissolving the dye (1 mg) in water (1 mL) followed by the addition of silica particles (50 mg). Following constant stirring at room temperature for 2 days, the suspension (100 μ L) was added into an aqueous solution (1 mL) of copolymer **3** (20 mg mL⁻¹) to allow the latter to electrostatically adsorb onto the particle surface. A control experiment was also conducted by adding the suspension (100 μ L) into water (1 mL) in the absence of copolymer **3**. Both samples were subsequently dialyzed for 4 days, centrifuged and the precipitates were re-suspended in 1 M HCl (1 mL) for 5 min at room temperature. In the case of silica particles with a copolymer shell, the HCl treatment facilitates the detachment of the copolymer from the particle surface. The suspensions were subsequently centrifuged and the supernatants containing released TB were diluted (5 times) before running the UV-Vis absorption spectroscopy.

The drug release curve was constructed by measuring the amounts of tobramycin released from drug-loaded mesoporous silica particles *without* polymeric shell at various times. Silica particles were loaded with tobramycin by stirring an aqueous solution (100 μ L) of tobramycin (400 mg mL⁻¹) with silica particles (1 mg) for 3 days. The particles were centrifuged, washed with DI water and re-suspended in DI water (1 mL) with continuous shaking at 37 °C. At each measurement time point (*i.e.*, 20 min, 40 min, 80 min, 2 h, 4 h, 8 h, 24 h, 48 h and 96 h), the absorption spectrum of the

supernatant after centrifugation was measured using a UV-Vis absorption spectrometer. The absorbance of tobramycin at 210 nm was used to determine the concentration of the drug¹⁰⁹. The cumulative amount of tobramycin released at a particular measurement time was determined by summing the amount measured at that time and the cumulative amount measured at the last measurement time. After each absorption measurement, the silica particles, recovered by centrifugation, were re-suspended in DI water (1 mL) and shaken. At the next measurement time, the absorption spectrum of the supernatant was again recorded. The total amount of tobramycin released from 1 mg of silica particles was estimated by fitting a double exponential rise function to the cumulative release curve (Figure 1B) (*i.e.*, $y = -(A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t)) + C$, where C is the total amount of drug released). The experiment was repeated at least twice.

A similar experiment was conducted for copolymer **3**-coated silica particles loaded with drug. Since the copolymer absorbs in the same region as tobramycin, a HPLC instrument (Waters 2695 Separation Module) equipped with a PDA detector set at 210 nm (Waters 2996 PDA) was used instead to determine the presence of released drug. An isocratic mobile phase consisting of a 0.05 M sodium hydrogen phosphate buffer (pH = 10) was used. Chromatography was carried out at 25 °C on an Alltima™ C 18 5u 250 mm × 4.6 mm column and the compounds eluted at a steady flow of 0.75 mL min⁻¹. The retention time of tobramycin is ~ 61 min. For copolymer **3**-coated silica particles loaded with drug, no tobramycin peaks were observed after dialysis (4 days).

2.2.14 Drug release in the presence of LPS-coated polystyrene beads

A HPLC experiment was conducted to measure the amounts of tobramycin released from copolymer **3**-coated mesoporous silica particles loaded with drug when stirred together with LPS-coated polystyrene beads in water. The HPLC calibration curve was first constructed by measuring the peak area of tobramycin (peak at 60–70 min retention time) at various concentrations (*i.e.*, 0.5, 1.0, 1.5 and 2.0 mg mL⁻¹). The LPS-coated polystyrene beads were prepared following the previous procedure¹¹⁰. Basically, 10 mg polystyrene latex beads (~ 1 µm mean particle size) were suspended in an aqueous solution (1 mL) of lipopolysaccharide (2.0 mg mL⁻¹) for 20 min. The drug-loaded copolymer **3**-coated silica particles (containing 10 mg of silica particles) were subsequently added into the LPS-coated polystyrene solution and continuously stirred at 100 rpm at room temperature. At each measurement time point (*i.e.*, 5 min, 10 min, 15 min, 20 min, 40 min, 80 min, 2 h and 4 h), the HPLC peak of the supernatant after centrifugation was measured. The amount of released tobramycin was determined using the calibration curve. Three control experiments were conducted: (i) an aqueous mixture (1 mL) containing drug-loaded copolymer **3**-coated silica particles and free LPS (2.0 mg mL⁻¹) was stirred at 100 rpm for 2 h, (ii) an aqueous mixture (1 mL) containing drug-loaded copolymer **3**-coated silica particles and LPS-coated polystyrene particles was allowed to be still for 2 h without stirring and (iii) an aqueous mixture (1 mL) containing drug-loaded copolymer **3**-coated silica particles and amine-modified polystyrene latex beads (~ 1 µm mean particle size and without LPS coating) (containing 10 mg beads) was stirred at 100 rpm for 2 h.

2.2.15 Colony expansion and proliferation tests

Exponential growth phase *P. mirabilis* (ATCC 7002) and *B. subtilis* (ATCC 6051) were used. The liquid culture media was lysogeny broth (LB). The exponential growth phase was obtained by diluting an overnight culture 20 times and then cultured for a further 3 h. When an exponential growth phase was obtained, the OD₆₀₀ of the culture was 0.9 and the bacteria number was determined to be 10⁷ CFU mL⁻¹. The solid culture media was LB with 0.2 % glucose and 1 % or 4 % agar for *P. mirabilis*, and LB with 0.5 % agar for *B. subtilis*.

To demonstrate that copolymer **3**-coated silica particles in the *absence* of loaded tobramycin is non-toxic against swarmer *P. mirabilis*, LB-agar solid media (1 %) was first prepared in 10-cm diameter Petri dishes. The surface of the agar was divided into two equal parts. 0.8 mg of drug-free copolymer **3**-coated silica particles were evenly applied onto the left side of the agar using an L-shape spreader and dried for 30 min before a fresh exponential-growth phase *P. mirabilis* culture (1 µL) was inoculated at a point on the right side of the agar (particle-free zone) (see Figure 2.8A). The dish was dried for a further 30 min before incubation for 20 h at 37 °C.

To examine the effects of released tobramycin on swarmer *P. mirabilis*, the drug was first loaded and encapsulated within the silica particles. In this case, silica particles (1 mg) and tobramycin (400 mg mL⁻¹) were mixed in water (100 µL) and stirred for 3 days. The suspension was then centrifuged and the precipitate was re-suspended in water (100 µL) containing copolymer **3** (25 mg mL⁻¹). The suspension was centrifuged and the precipitate was re-suspended in fresh DI water to achieve a particle

concentration ranging from 1 to 0.25 mg mL⁻¹.

Solid LB-agar media (1 %) was prepared in 10-cm diameter Petri dishes. The surface of the agar was divided into two equal halves. An aqueous suspension of copolymer **3**-coated particles (200 µL) was applied on the left half surface of the agar using an L-shape spreader to achieve various amounts of deposited particles (*i.e.*, 0.2 - 0.05 mg of silica particles). The suspension was dried for 30 min before a fresh exponential-growth phase *P. mirabilis* culture (1 µL) was inoculated at a point on the right side of the agar (particle-free zone) (see Figure 2.8B and C). The dish was dried for a further 30 min before incubation for 20 h at 37 °C.

To demonstrate that the drug release is not triggered by immobile cells, hard (4 %) LB-agar was used. To prevent swarming *P. mirabilis* from undergoing differentiation, a harder agar (*e.g.*, 4 %) can be used^{52, 82}. It is worthwhile to note that differences in agar concentration do not significantly affect the inhibition zones and antibiotic gradient of free tobramycin⁸². An exponential growth phase *P. mirabilis* culture (800 µL) was evenly inoculated on the entire agar surface and dried for 4 h to form a layer of immobile bacteria (see Figure 2.8D). Drug-loaded silica particles (0.2 mg) with and without a copolymer shell were deposited on the left side of the agar. The dish was then incubated for 20 h at 37 °C.

For the swarmer *B. subtilis* experiment, an aqueous suspension (200 µL) of copolymer **3**-coated silica particles loaded with tobramycin (0.05 mg) was first applied on the left half surface of a (0.5 %) LB-agar and dried for 30 min¹¹¹⁻¹¹². A fresh exponential-growth phase *B. subtilis* culture (1 µL) was inoculated at a point on the

right side of the agar. The dish was dried for a further 30 min before incubation for 20 h at 37 °C.

All experiments were repeated at least four times and the observations were found to be reproducible.

2.2.16 AFM force spectroscopy

Si wafers were cleaned by sonication in acetone solvent for 30 min, rinsed with DI water and dried by N₂ gas. A thin superglue liquid film was applied on the surface of the Si wafer by spin-coating. LPS, LPA and copolymer **3** were made into thick films by freeze drying before they are attached onto the thin superglue film. The samples were left to further dry overnight, and any samples unattached were washed away by rinsing thoroughly with PBS buffer solution.

SiO₂ AFM probes (spherical with diameter of 650 nm $\pm$ 10 %) were purchased from TipsNano. A similar method as used for modifying silica particles was used to functionalize the SiO₂ AFM probe with a shell of copolymer **3**. The modified probe was washed with PBS buffer to remove any unattached copolymer. The spring constants of bare probe and modified probe were calibrated and values of 3.6 and 5.2 N m⁻¹, respectively, were calculated by the built-in thermal tune. The force curves were measured in PBS buffer solution using an AFM instrument (Bruker, MultiMode 8). The adhesion forces between bare SiO₂ probe and copolymer **3**/LPS/LTA attached onto the wafer were measured. In addition, the adhesion forces between copolymer **3** adsorbed on the SiO₂ probe and LPS/LTA attached onto the wafer were also measured.

2.2.17 Surface charge neutralization by zeta potential study

An overnight *P. mirabilis* culture (50 μ L) was re-suspended in fresh LB media (5 mL) and continuously shaken at 180 rpm for 3 h at 37 °C. The culture was re-suspended in a phosphate buffer to afford OD₆₀₀ $\sim$ 0.2. 700 μ L of the bacterial culture was then used in the zeta potential experiment. Increasing amounts of copolymer **3** were subsequently added and the resulting zeta potentials measured. A similar procedure was also used to measure the zeta potentials of *B. subtilis* in the presence of varying amounts of copolymer **3**.

2.2.18 Wide-field fluorescence microscopy setup

The wide-field fluorescence microscopy consists of an inverted microscope (IX 71, Olympus) coupled to a highly sensitive CCD camera (CascadeII 512B, Photometrics). A white light source was fixed above the bacteria sample and used to observe bacteria motion and a 633 nm HeNe laser source (35 mW, Melles Griot) was used for fluorescence microscopy. The laser light was circular polarized and filtered by an excitation filter (Z633/10, Chroma) before being focused onto the back-focal plane of the objective lens. The transmitted light and fluorescence from the sample were passed through an air objective lens (20 $\times$, N.A. = 0.40, Olympus), a dichroic mirror (Z633rdc, Chroma), an emission filter (HQ645lp, Chroma) and a 3.3 $\times$ camera lens before entering the CCD camera. The dimension of an image frame was measured to be 120 $\times$ 120 μ m² (512 $\times$ 512 pixels) using a stage micrometer and the rate of the CCD camera is 30 frames per second.

An aqueous solution (20 μL) of copolymer **3** (25 mg mL^{-1}) was mixed with an aqueous suspension (10 μL) of drug-free silica particles (25 mg/mL) and incubated at room temperature for 30 min before centrifugation. The precipitate was washed using water and re-suspended in water (1 mL). A drop of the particle suspension (1 μL) was deposited on the surface of 1 or 0.5 % LB-agar and dried for 30 min. A freshly prepared *P. mirabilis* exponential growth phase culture (1 μL) was inoculated close to the particle zone on the 1 % agar. The agar was dried for 30 min before incubation at 37 °C for 10 h to form a swarmer colony. For swarmer *B. subtilis*, the cells were inoculated close to the particle zone on the 0.5 % agar and dried for 30 min before incubation at 37 °C for 3 h to form a swarmer colony. Wide-field fluorescence microscopy was used to visualize the interaction between dye-labelled copolymer chains on the particle surface and motile swarmer *P. mirabilis* and *B. subtilis*.

2.2.19 Video description

Video 1, 2 and 3 can be watched on the website (<https://www.nature.com/articles/s41467-018-06729-6#Sec29>). Please see Supplementary movie 1, 2 and 3 (corresponding to video 1, 2 and 3, respectively) in the section of Electronic supplementary materials.

Video 1. Continuous wide-field fluorescence images of swarmer *P. mirabilis* cultured on an (1 %) agar in the presence of mesoporous silica particles with dye-labelled copolymer **3** attached on the surface. The particles are not loaded with drug. In this video, white light is used to image the bacterial colony and red light (633 nm light) is

used to image dye-labeled copolymer 3. The illumination source was alternated between white light and red light with white light illumination turned on at 0.588, 4.475, 9.604 and 15.909 s, and red light at 2.646, 7.416 and 11.989 s. During red light illumination, bright swirling streaks are observed which are due to detached copolymer 3 moving within the swarmer colony. During the transition from one light source to the other, a superposition of the images of the colony (from white light) and dye-labeled copolymer 3 (from red light) can be observed. The dimension of the image is $120 \times 120 \mu\text{m}^2$.

Video 2. Continuous wide-field fluorescence images of multi-layered swarmer *B. subtilis* cultured on an (0.5 %) agar in the presence of mesoporous silica particles with dye-labelled copolymer 3 attached on the surface. The particles are not loaded with drug. In this video, white light is used to image the bacterial colony and red light (633 nm light) is used to image dye-labeled copolymer 3. The illumination source was alternated between white light and red light with red light illumination turned on at 0.561, 11.473, 20.868 and 31.615 s, and white light at 9.791, 15.527 and 25.220 s. During red light illumination, no obvious bright streaks corresponding to copolymer 3 chains being carried away from the particles are seen. The dimension of the image is $120 \times 120 \mu\text{m}^2$.

Video 3. Continuous wide-field fluorescence images of single-layered swarmer *B. subtilis* cultured on an (0.5 %) agar in the presence of mesoporous silica particles with dye-labelled copolymer 6 attached on the surface. The particles are not loaded with drug. In this video, white light is used as the illumination source for the first 3 s, and a

typical single-layered swarmer colony is seen. At 3 s, the illumination source is switched to a 633 nm light that excites Atto 647N molecules. No obvious bright streaks corresponding to copolymer chains being carried away from the particles are seen. The dimension of the image is $120 \times 120 \mu\text{m}^2$.

2.3 Results and discussion

2.3.1 Copolymer 3 seals in payloads

Copolymer **3** was prepared by atom-transfer radical polymerization of NIPAAm and AEMA with ethyl-2-bromopropionate as initiator and CuBr as catalyst to yield copolymer **1** with $M_n = 10171 \text{ g mol}^{-1}$ and $M_w = 21985 \text{ g mol}^{-1}$ (Figure 2.2)¹¹³. A fraction of the amine groups in **1** ($\sim 20 \%$) were converted to maleimides by reacting with 3-maleimidopropionic acid to give copolymer **2**. Peptide ligands YVLWKRKRKFCFI-NH₂ were subsequently attached to **2** *via* a stable carbon (maleimide)-sulphur (cysteine) bond to obtain the desired copolymer **3** with $M_n = 14212 \text{ g mol}^{-1}$ and $M_w = 43033 \text{ g mol}^{-1}$ (Figure 2.2). The average number of NIPAAm, AEMA and peptide units per chain is $m \sim 77$, $n \sim 11$ and $p \sim 2$, respectively. Copolymer **3** is positively charged with a zeta potential $\zeta_{\text{polymer}} = 21.8 \pm 1.3 \text{ mV}$ due to the presence of the remaining amine groups. The lower critical solution temperature LCST of copolymer **3** is determined to be 53°C ¹¹⁴ which is significantly higher than the temperature used in the experiments reported here ($\leq 37^\circ\text{C}$). The mesoporous silica particles (average diameter = $264 \pm 60 \text{ nm}$), with an average mesopore diameter of 3.5 nm , were prepared using standard

procedures (Figure 2.4)¹⁰⁶.

In order to show that copolymer **3** is able to seal in payloads, toluidine blue (TB) was first loaded into mesoporous silica particles, and the copolymer was electrostatically bound to the negatively charged particle surface (*i.e.*, measured zeta-potential of MCM-41 $\zeta_{\text{MCM-41}} = -41.0 \pm 0.9$ mV) to form a copolymer shell. TB is a positively charged dye that can be readily quantified using spectroscopic methods such as UV-vis absorbance. The strong absorbance of TB at visible wavelengths makes it a convenient indicator to quickly assess the loading ability of the dye into polymer-coated silica particles. The dye-loaded particles were dialyzed for 4 days before treatment with 1 M HCl (5 min) to remove copolymer **3** chains from particle surface. A similar experiment was also conducted on dye-loaded silica particles but without an initial copolymer shell. For the latter, the absorption spectrum of TB released during the HCl treatment has low absorbance intensity (Figure 2.5A); indicating that most of the dye was released after 4 days of dialysis and only a small amount retained within the particles. On the other hand, for dye-loaded particles with an initial copolymer shell, a significant amount of TB was released upon detachment of copolymer chains from the particle surface during the HCl treatment (Figure 2.5A). In particular, for the 635 nm absorption peak, the absorbance of TB released from particles with an initial copolymer shell is ~ 18 times larger than that in the absence of an initial shell. This clearly suggests that during the dialysis period, copolymer **3** is able to prevent leakages of TB stored within the pores of the silica particles. We therefore propose that copolymer **3** is effective for sealing in active compounds loaded into mesoporous silica particles.

Figure 2.4 (A) The TEM image of MCM-41 silica particles. (B) The zoomed-in TEM image showing the mesoporous structure of MCM-41 silica particles. The pore size is 3.5 nm. Inset of (B) shows the histogram of the size (diameter) distribution of the particles. The mean diameter obtained from a Gaussian distribution fit is 264 ± 60 nm.

Figure 2.5 Loading and release of toluidine blue (TB) and tobramycin. (A) Absorption spectra of TB released after 4 days of dialysis and 1 M HCl treatment for mesoporous silica particles with (red) and without (black) an initial copolymer shell. (B) Drug release curve showing the amount of tobramycin released from drug-loaded mesoporous silica particles without copolymer shell at various times. The release curve for the first 8 h is given in the inset.

In this study, the drug used is tobramycin which is an antibiotic known to be active against *P. mirabilis*¹¹⁵⁻¹¹⁶. From UV-vis absorption spectroscopy, the release curve of tobramycin from silica particles without a copolymer shell after the drug has been loaded into the particles is given in Figure 2.5B. By fitting the release curve to a double-exponential rise function, the maximum amount of tobramycin released from the particles is estimated to be ~ 0.2 mg of drug/mg of particles which is comparable to the loaded amount of tobramycin in mesoporous silica particles (*i.e.*, 0.234 ± 0.006 mg of drug/mg of particles). We note that the antibiotic released from silica particles follows a two-step process; a fast release within the first 2 h followed by a slower release. In the case of copolymer **3**-coated silica particles containing the drug, no detectable amount of tobramycin was released after dialysis (4 days); indicating that copolymer **3** prevented leakages of tobramycin from the particles.

Figure 2.6 Amount of tobramycin released vs. square-root of time. The correlation coefficients of the linear fits for the first and second steps are $R_c = 0.98$ and 0.97 , respectively.

We used the Higuchi model to describe the drug release kinetics¹¹⁷. This model, based on a diffusion controlled release of drug from a matrix system, is commonly utilized to describe the kinetics of drug release from mesoporous silica¹¹⁸. According to the Higuchi model, the amount of drug released after time t is given by Q :

$$Q = \sqrt{\frac{D\varepsilon}{\tau}(2C - \varepsilon C_s)C_s t}$$

where D is the diffusion coefficient of the drug in the matrix solution, ε is the porosity of the matrix, τ is the capillary tortuosity factor, C is the total amount of drug in the matrix and C_s is the solubility of drug in the matrix solution. Therefore, for a diffusion controlled release of drug, Q displays a linear relationship with $t^{1/2}$.

Figure 2.6 shows a plot of Q vs. $t^{1/2}$ for the release of tobramycin based on Figure 2.5B. We note from Figure 2.6 that the antibiotic released from silica particles follows a two-step process; a fast release within the first 2 h followed by a slower release. For both steps, the Q vs. $t^{1/2}$ plot can be described by a linear relationship, suggesting a diffusion controlled kinetics for the release of tobramycin. The last point in Figure 2.6 at $t^{1/2} = 9.8 \text{ h}^{1/2}$ shows slight deviation from the linear fit since it is close to the time region when the amount of drug released has reached steady-state. The first (fast) step is likely due to the release of drug molecules that are either weakly bound to the silica pore surfaces or not efficiently encapsulated within the matrix¹⁰⁸, whereas the second (slower) release step is due to drug molecules that interact relatively stronger with silica due to the amine groups on tobramycin. There is also a possibility of a small fraction the drug that is still tightly bound to the surface of the silica after a long time and remains trapped in the pores. Another plausible explanation of the two-step process could be due to

different dissolution rates of silica.

2.3.2 Interaction between LPS-coated polystyrene and copolymer 3-coated silica particles

MIMS is first demonstrated in water using an LPS-coated polystyrene (PS) bead. In this case, PS latex beads ($\sim 1\ \mu\text{m}$ mean particle size) were first treated with LPS to allow the adsorption of LPS onto the surface of the beads. When an aqueous mixture of drug-loaded copolymer **3**-coated silica particles (10 mg) and free LPS ($2\ \text{mg mL}^{-1}$) was stirred at 100 rpm for 2 h, no released drug was detected *via* HPLC. On the other hand, when a mixture of drug loaded copolymer **3**-coated silica particles and LPS-coated PS beads (10 mg) was stirred at 100 rpm to enable the two entities to come into contact with each other, tobramycin was released and the drug release kinetics is given in Figure 2.7. We note that *ca.* 0.15 mg of drug/mg of particles was released after stirring with LPS-coated PS latex beads which is in agreement with the amount of drug released from silica particles without an initial copolymer shell (Figure 2.5B). It is also observed that in the absence of stirring, no released drug was detected in still water.

Figure 2.7 Drug release curve showing the amount of tobramycin released from drug-loaded mesoporous silica particles covered with a coat of copolymer **3** at various times. The drug is released when LPS-coated polystyrene latex beads are stirred with the silica particles at 100 rpm. The amount of drug released was determined using HPLC.

We propose that LPS on PS beads binds to copolymer **3** on silica particles when the two entities are in close contact. The copolymer is subsequently pulled off the surface of silica particles when the particles and PS beads are forced apart during stirring. The release of tobramycin occurs at a faster rate (> 80% of steady-state amount released within 2 h) compared to the release of drug in Figure 2.5B due possibly to more collisions occurring between the exposed silica particles and PS beads. Free LPS by itself is unable to cause detachment of copolymer **3** from silica particles due to insufficient stripping energy. When a mixture of amine-modified PS beads ($\sim 1\ \mu\text{m}$, 10 mg) that are *not* coated with LPS and drug-loaded copolymer **3**-coated silica particles

was stirred at 100 rpm, no significant amount of released drug was detected even after 2 h; suggesting that in the absence of LPS on PS beads, MIMS is not in operation. Furthermore, collision between PS beads and silica particles alone does not lead to the detachment of copolymer chains from the latter.

2.3.3 Copolymer 3-coated silica particles containing drug inhibits swarmer *P. mirabilis* colony expansion on agar surface.

The efficacy of the I-DRS against the expansion of swarmer *P. mirabilis* is studied next. Drug-free copolymer 3-coated silica particles (Figure 2.8) and copolymer 3-coated particles containing tobramycin (Figure 2.8B and C) were separately deposited on the left side of the (1 %) agar surface. The cells were subsequently inoculated on the right side of the agar and cultured for 20 h at 37 °C. An expanding swarmer colony was achieved after a lag time of ~ 8 h. When 0.8 mg of drug-free copolymer 3-coated silica particles were used, the swarmer cells colonized the entire agar surface (Figure 2.8A). This suggests that in the absence of drug, the I-DRS is non-toxic when the amount used is as high as 0.8 mg.

When copolymer 3-coated silica particles loaded with tobramycin (*ca.* 0.2 mg of drug/mg of particles) were used, the swarmer colony spread throughout the particle-free surface (*i.e.*, right side) of the agar (Figure 2.8B and C). However, an impediment to the expansion of the colony across the surface of the agar containing the I- DRS (*i.e.*, left side) was observed. In addition, the swarmer colony was found to possess a secondary front (thin layer) that preceded a primary front (thick layer). Two parameters

are employed to characterize the I-DRS efficacy: L_s and L_p are the distances from the center of the vertical line dividing the particle (left side) and particle-free (right side) zones to the intersection points between the horizontal bisector and the secondary and primary fronts, respectively. When 0.2 (Figure 2.8B) and 0.05 (Figure 2.8C) mg of silica particles were used, the secondary fronts of both colonies expanded only slightly into the particle zone with $L_s = 3.9$ and 13.5 mm, respectively. On the other hand, both the primary fronts were not able to cross into the particle zone; $L_p = 7.1$ and 2.3 mm for 0.2 and 0.05 mg of particles, respectively. Therefore, when swarmer cells from the secondary front enter into the particle zone and come in contact with the particles, drug is released. Bacteria in the secondary front are subsequently damaged and the colony is not able to continue its expansion. In the presence of a larger amount of particles, more tobramycin is released into the colony resulting in a greater impediment to colony expansion (*i.e.*, shorter L_s and longer L_p for 0.2 mg particles compared to 0.05 mg particles). It is worth mentioning that tobramycin released from the silica particles deposited on the left side of the agar penetrates through agar, however, a concentration gradient is expected such that the amount of free tobramycin that has diffused away to the right-side of the agar is likely to be lower than the minimum inhibitory concentration (MIC) against *P. mirabilis* ($\sim 2 \mu\text{g mL}^{-1}$) (Figure 2.9), and does not cause inhibition of the proliferation of cells on that side of the agar.

Figure 2.8 Release of drug *via* MIMS. Colony expansion (A-C) and proliferation (D) of *P. mirabilis* observed 20 h after cells were inoculated on 1 % (A-C) and 4 % (D) agar. Copolymer **3**-coated silica particles were deposited evenly on the left side of the agar surface. 0.8 mg of drug-free particles (A), and 0.2 (B, D) and 0.05 (C) mg of particles containing tobramycin were used. (A-C) *P. mirabilis* cells were inoculated on the right side of agar at point x. The vertical dotted line (black) is the boundary between the particle and particle-free zones that divides the agar into two equal halves, and the horizontal dotted line (green) in (A) bisects it. The blue and red arrows in (B) and (C) indicate the intersection points between the horizontal bisector with the secondary and primary colony fronts, respectively. (D) *P. mirabilis* were inoculated on the whole agar and immobile *P. mirabilis* cells were seen to proliferate throughout the agar.

Figure 2.9 Tobramycin E-test of *P. mirabilis* in the absence (A) and presence (B) of copolymer **3**.

A tobramycin E-test assay was conducted to show that for *P. mirabilis*, the MIC value of tobramycin against the bacteria is unaffected by the presence of copolymer **3**. Using a tobramycin E-test strip, the MIC value of tobramycin against *P. mirabilis* on agar is determined to be $\sim 2.0 \mu\text{g mL}^{-1}$ (Figure 2.9A); in agreement with previously reported values¹¹⁹. In a separate experiment, *P. mirabilis* was applied onto an agar whose entire surface was first treated with 0.28 mg of copolymer **3** (*i.e.*, approximately twice the amount of copolymer **3** attached onto the silica particles deposited on the left side of the agar in Figure 2.8B). In this case, the MIC value of tobramycin against *P. mirabilis* exposed to copolymer **3** is determined to be also $\sim 2.0 \mu\text{g mL}^{-1}$ from the tobramycin E-test strip (Figure 2.9B); indicating that the bacteria do not become more susceptible nor the activity of the antibiotic enhanced in the presence of the copolymer used here.

A control experiment was also performed to examine the effects of immobile cells on the I-DRS. In this case, *P. mirabilis* cells were inoculated on the entire surface of a hard (4 %) agar containing, on the left side surface, 0.2 mg of copolymer **3**-coated silica particles loaded with tobramycin (*ca.* 0.2 mg of drug/mg of particles). After the cells

were dried and cultured for 20 h (at 37 °C), it was seen that *P. mirabilis* proliferated into a thick layer of immobile (vegetative) cells throughout the agar with no obvious inhibition of cell growth at the side of the surface containing the particles (Figure 2.8D). When a similar experiment was performed using drug-loaded silica particles *without* a copolymer shell, the leakage of tobramycin into the surroundings prevented the proliferation of bacteria in the particle-zone (Figure 2.10). Since differences in agar concentration do not significantly affect the inhibition zones and antibiotic gradient⁸², the absence of bacterial inhibition zones in Figure 2.8D indicates that tobramycin is not released by immobile bacteria and this is in agreement with our earlier findings that when copolymer chains are not stripped off the particle surface, minimal leakage of payload occurs (see Figure 2.5A).

Figure 2.10 *P. mirabilis* cells were inoculated throughout the surface of a (4 %) agar and dried. Silica particles (0.2 mg) loaded with tobramycin but without a copolymer shell are deposited on the left half. The bacteria proliferated at the side of the agar that does not contain any particles (*i.e.*, opaque right half) but not at the side containing drug-loaded particles (*i.e.*, significantly less opaque left half). In the absence of the copolymer shell, tobramycin is easily released from the silica particles into the agar. This experiment proves that the dosage of drug containing silica particles is large enough to inhibit proliferation of *P. mirabilis*.

Despite the antimicrobial effects of YVLWKRKRKFCFI-NH₂ against *P. mirabilis* (MIC = 1.6 mg mL⁻¹, Figure 2.11), Figure 2.8A shows that the peptide from the I-DRS does not cause obvious damage to the swarmer colony; probably because of its low concentration (< MIC). Furthermore, using an E-test assay, it is demonstrated that the MIC of tobramycin against vegetative *P. mirabilis* (~ 2.0 µg mL⁻¹) remained unchanged in the presence of a comparable amount of copolymer 3 as utilized in Figure 2.8B (Figure 2.9). Therefore, the bacteria do not become more susceptible nor the activity of the antibiotic enhanced in the presence of the copolymer used.

Figure 2.11 (A) *P. mirabilis* bacterial colonies on agar plate after treatment with various concentrations of peptide. (B) Relative bacterial growth after treatment with various concentrations of peptide with respect to growth in the absence of peptide. The curve was fit to a triple-exponential decay function and the minimum concentration of peptide needed to give 90 % growth inhibition is 1.6 mg mL^{-1} .

2.3.4 Copolymer 3 adheres stronger to LPS than to silica

AFM force spectroscopy experiments were conducted to determine the adhesion forces between copolymer **3** and SiO_2 and LPS. It is observed that the interaction between a bare SiO_2 spherical tip and a layer of LPS (from *P. mirabilis*) attached on the surface of a Si wafer is negligible (see Figure 2.12 for a representative force curve). On the other hand, significant interaction between the bare SiO_2 tip and a layer of copolymer **3** on a Si wafer is observed, where a representative force curve in Figure 2.13A yields an adhesion force of 1.26 nN. A histogram of the adhesion force between the bare SiO_2 tip and copolymer **3**, based on 100 force curves (inset of Figure 2.13A), shows that the adhesion force ranges from 0.78 to 2.94 nN.

Figure 2.12 Representative force curves plotted for the interactions between a bare SiO₂ spherical tip and a layer of LPS (from *P. mirabilis*) (a) and LTA (from *B. subtilis*) (b) attached on a Si wafer. A representative force curve plot for the interaction between a copolymer **3** functionalized SiO₂ tip and a layer of LTA attached on a Si wafer (c).

In a separate experiment, a SiO₂ spherical tip functionalized with a shell of copolymer **3** was used to determine the adhesion force between the copolymer and LPS. We observed a relatively large adhesion force between the modified SiO₂ tip and LPS as illustrated by 3 representative force curves collected before 90 ramp cycles in Figure 2.13B. The adhesion peaks are broad due to the stretching of outer copolymer upon interaction with LPS on the Si wafer. A histogram of adhesion force, based on 90 force curves, indicates that the adhesion force between copolymer **3** and LPS ranges between 3.26 and 26.92 nN (inset of Figure 2.13B). For the 90 to 432 ramp cycles, the adhesion peaks for LPS become narrower and a much smaller force is seen (*e.g.*, 1.7 to 3.5 nN for 3 representative curves in Figure 2.14). After 432 ramp cycles, no adhesion peak is observed (Figure 2.14). This is likely due to the peeling off of copolymer **3** from the

contact surface of the SiO₂ tip as a result of the stronger adhesion force between the copolymer and LPS. We, therefore, propose that copolymer **3**, initially adsorbed on the surface of silica particles, will adhere more strongly to swarmer *P. mirabilis* in the secondary front when the latter comes into contact with the silica particles. The kinetic energy from the motile bacteria is able to strip the copolymer off the surface of the silica particles (*i.e.*, MIMS is in operation) as demonstrated experimentally in the wide-field fluorescence microscopy (WFFM) measurements below.

Figure 2.13 AFM force spectroscopy. (A) A representative force curve plotted for the interaction between a bare SiO₂ spherical tip and a layer of copolymer **3** attached on a Si wafer. The adhesion force histogram for 100 force curves is given in the inset. (B) Representative force curves plotted for the interaction between a copolymer **3** functionalized SiO₂ tip and a layer of LPS attached on a Si wafer and collected before 90 ramp cycles (adhesion forces for curves 1, 2 and 3 are 17.07, 16.36 and 7.10 nN, respectively). The adhesion force histogram before 90 ramp cycles is given in the inset.

Figure 2.14 Representative force curves plotted for the interaction between a copolymer **3** functionalized SiO₂ tip and a layer of LPS (from *P. mirabilis*) attached on a Si wafer and collected 90 to 432 ramp cycles (adhesion forces for curves 1, 2 and 3 are 1.67, 3.60 and 3.18 nN, respectively) (A), and after 432 ramp cycles where no adhesion peak is seen (B).

2.3.5 Real-time monitoring of MIMS using WFFM

In the WFFM study, copolymer **3**, labelled with a photostable Atto 647N chromophore (ATTO-TEC GmbH), is electrostatically bound to drug-free particles. Video 1 (see in Section 2.2.19) shows a typical swarmer *P. mirabilis* colony expanding from left to right on an (1 %) agar containing copolymer **3**-coated silica particles. Copolymer **3** chains were detached from silica particles found inside the swarmer colony (*i.e.*, white fluorescent streaks flowing away from particles and swirling inside the colony) and not from particles located outside the colony. An example is given in Figure 2.16 for the particle at ($x = 50 \mu\text{m}$, $y = 83 \mu\text{m}$). An overlay of the images of the swarmer colony and labeled copolymer at time $t = 0.59 \text{ s}$ is seen in Figure 2.15A where the colony front is in contact with the particle. As the colony continues to expand, copolymer **3** is stripped

off the particle surface by the swarmer cells (*i.e.*, at $t = 3.92$ s where only the silica particle is imaged with no image interference from the colony (Figure 2.15B) and at $t = 4.48$ s where images of both the colony and particle are recorded (Figure 2.15C)). As time progresses, more copolymer chains are removed and swirls of fluorescent white streaks arising from the transportation of removed copolymers by the motile cells are visible (Figure 2.15D). When the cells are immotile (Figure 2.8D), MIMS is not in operation which prevents both the release of drug and inhibition of cell proliferation.

Figure 2.15 Visualizing MIMS in real-time. Wide-field fluorescence microscopy images of a dye-labelled copolymer **3**-coated silica particle at ($x = 50 \mu\text{m}$, $y = 83 \mu\text{m}$) in Video 1 (Section 2.2.19). (A) and (C) show the overlap of the swarmer colony and labelled copolymer **3** recorded with white and red light irradiation, respectively, and (B) and (D) show only the images of copolymer **3**. Examples of fluorescent streaks arising from removed copolymer **3** are enclosed within blue dotted lines in (B) and (D). The time of each image t is given. The image contrast is enhanced resulting in particles appearing larger than their actual size due to light scattering.

2.3.6 MIMS is not in operation against swarmer *B. subtilis* colony.

The MIC value of tobramycin against vegetative Gram-positive *Bacillus subtilis* is $\sim 1.5 \mu\text{g mL}^{-1}$ (Figure 2.16) which is slightly lower than the MIC against *P. mirabilis*. An experiment similar to Figure 2.8C was therefore conducted on swarmer *B. subtilis* to

compare its effects on the I-DRS. In this case, the colony is capable of expanding fully into the left side of an (0.5 %) agar containing 0.05 mg of drug-loaded copolymer **3**-coated silica particles on the agar surface (Figure 2.16); suggesting that no lethal amounts of drug are released from the particles despite the presence of motile *B. subtilis*.

Figure 2.16 (A) Tobramycin E-test of *B. subtilis*. (B) Colony expansion of *B. subtilis* observed 20 h after cells were inoculated on 0.5 % agar at point x. 0.05 mg of copolymer **3**-coated silica particles containing tobramycin were deposited evenly on the left side of the agar surface.

Figure 2.17 ITC thermogram of interactions between LTA (from *B. subtilis*) and the YVLWKRKRKFCFI-NH₂. From the ITC thermogram and fitting the ITC data into a single set of binding sites, a K_a value of $0.042 \mu\text{M}^{-1}$ is obtained.

Figure 2.18 Copolymer 3 binds more to *P. mirabilis*. Changes of zeta potential of *P. mirabilis* and *B. subtilis* cells membrane in the presence of copolymer 3.

Weaker binding is observed between the peptide YVLWKRKRKFCFI-NH₂ and lipoteichoic acid (LTA) found on the cell walls of *B. subtilis* ($K_a = 0.042 \mu\text{M}^{-1}$, Figure

2.17). In addition, a surface charge neutralization by zeta potential study was performed. In the absence of copolymer **3**, *P. mirabilis* exhibits a larger negative potential (~ -20 mV) as compared to *B. subtilis* (~ -4 mV). As shown in Figure 2.18, the addition of *ca.* 1-2 μM of copolymer **3** neutralizes the negative charge of the bacterial cell wall. A larger over compensation of surface charges is seen for *P. mirabilis* (*e.g.*, zeta potentials of +7 mV and +2 mV for *P. mirabilis* and *B. subtilis*, respectively, are obtained when the concentration of copolymer **3** added is 8 μM). This means that for a given concentration of copolymer **3** added, the number of copolymer **3** bound to *P. mirabilis* is significantly higher than that for *B. subtilis*.

The AFM force curves for interactions between bare SiO_2 spherical tip and LTA, and between LTA and copolymer **3** adsorbed on a SiO_2 spherical tip do not display adhesion peaks (Figure 2.14); suggesting that the adhesion force between copolymer **3** and LTA is too weak to be measured by AFM. We, therefore, propose that when swarmer *B. subtilis* come into contact with the copolymer on silica particles, the copolymer remains attached onto the particles. Since the copolymer is not adhered to motile *B. subtilis*, the motile cells are unable to strip the copolymer off the silica particle surface. This mechanism is in line with the WFFM study that shows a distinct absence of bright streaks moving away from drug-free silica particles coated with dye-labelled copolymer **3** within a swarmer *B. subtilis* colony (Video 2 in section 2.2.19 for multi-layer colony and S3 for single-layer colony)¹¹¹⁻¹¹². This demonstrates two points: (i) collision by fast moving bacteria is not sufficient to cause the electrostatically bound

copolymers to be detached from the silica particle surface, and (ii) MIMS is not in operation here.

2.4 Conclusion

In summary, the I-DRS proposed in this study releases therapeutic drugs upon sensing the presence of swarmer *P. mirabilis* and is able to effectively impede the spread of the colony. An important feature of the I-DRS is its affinity to *P. mirabilis* due to the relatively strong binding between the conjugated copolymer-(YVLWKRKRKFCFI-NH₂) system with the Gram-negative bacteria. Further studies are underway to realize the applications of MIMS in biomedicine where the short and long term toxicological effects of the I-DRS in humans are noted¹²⁰⁻¹²¹.

Chapter 3 Increasing Antibiotic Activity by Rapid Bioorthogonal Conjugation of Drug to Resistant Bacteria Using an Upconverted Light-activated Photocatalyst[†]

Abstract

The antibiotic vancomycin (Van) is often used as the drug of last resort to treat methicillin resistant *Staphylococcus aureus*. Due to the emergence of Van-resistant microbes, it is necessary to continuously design strategies to increase the efficacy of Van against resistant cells. In this study, an efficient method of bio-conjugating Van to bacteria is proposed using near-infrared (NIR)-light activation. A Nd³⁺-sensitized upconversion nanocrystal (UCNC) decorated with toluidine blue O (TB) on its surface undergoes upconverted energy transfer from the UCNC to TB when excited by 808 nm light. The photoexcited TB then catalyses the conversion of the dihydrotetrazine (dHTz) moiety in a Van-dHTz conjugate system to tetrazine which undergoes efficient inverse electron demand Diels-Alder reaction with prior attached norbornene molecules on bacterial cell walls. The enhanced affinity of Van to bacteria by covalent bonding improves the activity of the drug against drug-resistant Enterococci, and the MIC is reduced by 6- to 7-fold as compared to neat Van. We demonstrate that the mode of action is due to increased inhibition of peptidoglycan cell wall biosynthesis. The findings in this study demonstrate that on-demand NIR-light activated bioorthogonal conjugation of Van to microbes is a viable alternative treatment in combating drug-

resistant bacteria.

[†] This chapter is adapted from Du, Q.; Wu, X.; Bi, W.; Xing, B.; Yeow, E.K.L., Increasing antibiotic activity by rapid bioorthogonal conjugation of drug to resistant bacteria using an upconverted light-activated photocatalyst. *Journal of Materials Chemistry B*, **2021**, 9, 3136.

3.1 Introduction

Ultraviolet (UV)-visible light have conventionally been utilized in photochemistry to either directly excite the reaction substrate or photocatalyst in order to induce reactions (*e.g.*, in aryl cross-coupling reactions)¹²²⁻¹²⁴. Unfortunately, for biological applications, UV light is not ideal because it damages the DNA of cells and causes autofluorescence. In addition, UV-visible light suffers from poor tissue penetration depth. In order to circumvent these problems, near-infrared (NIR) photochemistry has recently gained much attention¹²⁵⁻¹²⁷. Upconversion nanocrystals (UCNC) consisting of a NaYF₄ host co-doped with lanthanide ions such as Nd³⁺, Yb³⁺, and Tm³⁺ or Er³⁺ are capable of converting NIR light to UV-visible light¹²⁸⁻¹²⁹ which is then used to drive photochemical reactions such as photocoupling¹³⁰, photopolymerization¹³¹⁻¹³², photoisomerization¹³³ and photolysis¹³⁴.

An important but often neglected area in photochemistry is the development of NIR light-activated photocatalysts (PC) that can allow one to access a wider

range of chemical reactions¹³⁵⁻¹³⁷. Recently, we have prepared ultrathin KNdF₄ nanoplates that efficiently Lewis-acid-catalyzed the addition of trimethylsilyl cyanide to various ketones in the presence of NIR activated photothermal heat¹³⁸. Since several organic and transition-metal based photocatalysts absorb UV-visible light, a system consisting of the PC grafted onto the surface of UCNCs is a promising method of photo-activating the PC via NIR light. In this way, upconverted photons from the UCNC excite the PC which can then catalyse reactions. Unfortunately, such studies are scarce. A work that we are aware of was reported by Freitag and co-workers who demonstrated the oxidation of ethyl-2-bromo-2-phenylacetate using a conjugated UCNC-[Ru(bpy)₃]²⁺ photoredox catalyst¹³⁹.

The antibiotic vancomycin (Van) is often used as the drug of last resort to treat methicillin resistant *Staphylococcus aureus* (MRSA). A tight binding complex formed between Van and the -D-Ala-D-Ala termini of the bacterial cell wall peptidoglycan results in cell growth inhibition and eventual cell lysis. For Van-resistant bacteria, the pathogens remodel their cell walls and convert the termini -D-Ala-D-Ala to -D-Ala-D-Lac. This results in a sharp drop in the binding affinity between Van and the resistant bacteria, and a significant reduction in drug efficacy. Due to the emergence of Van-resistant pathogens and decline in the number of effective drugs, it is necessary to continuously design various strategies to increase the efficiency of Van against resistant cells. Several groups have reported novel Van derivatives¹⁴⁰⁻¹⁴¹, Van dimers¹⁴²⁻¹⁴³ and Van

attached to bacterial targeting ligands¹⁴⁴⁻¹⁴⁶ as promising candidates to combat drug-resistant bacteria due to their enhanced affinity to the cells.

In this study, we aim to photo-catalyse the conjugation reaction between Van and bacterial cell walls using NIR light in order to increase the affinity of the drug and enhance its activity. To this end, a photocatalyst comprising of a Nd³⁺-sensitized core-shell UCNC whose surface is decorated with toluidine blue O dye molecules (TB) (*i.e.*, TB-UCNC) is used. The conjugate TB-UCNC is suitable for biological applications since the 808 nm excitation light used displays good tissue penetration¹⁴⁷ and avoids overheating of biological samples¹²⁹. To achieve bioorthogonal reaction, the efficient inverse electron demand Diels-Alder (IEDDA) reaction between tetrazine (Tz) and strained dienophile is utilized¹⁴⁸. On-demand conjugation by NIR light activation is achieved when Van is initially linked to an unreactive dihydrotetrazine (dHTz) to form a Van-dHTz conjugate system (Scheme 3.1A). The dHTz moiety undergoes photocatalytic oxidation to the reactive Tz (Scheme 3.1A) by TB-UCNC; forming Van-Tz¹⁴⁹. The Van-Tz can bind to bacteria whose cell walls are pre-functionalized with norbornene molecules (Nb) *via* IEDDA (Scheme 1B). The antibacterial activity of our proposed method against Gram-positive *Bacillus subtilis* (*B. subtilis*), and Van resistant *Enterococcus faecium* (VanA) and *Enterococcus faecalis* (VanB) is examined here.

Scheme 3.1 (A) Chemical structures of **1a**, **1b**, **Van**, **Van-dHTz** and **Nb-DBCO**.

(B) Scheme illustrating the bioorthogonal conjugation of **Van-dHTz** to **Nb**-functionalized bacterial cell wall in the presence of **TB-UCNC** and **808 nm** light excitation.

3.2 Experimental section

3.2.1 Materials

Yttrium (III) acetate hydrate ($\text{Y}(\text{CH}_3\text{CO}_2)_3 \cdot x\text{H}_2\text{O}$, 99.9%), ytterbium (III) acetate hydrate ($\text{Yb}(\text{CH}_3\text{CO}_2)_3 \cdot x\text{H}_2\text{O}$, 99.9%), erbium (III) acetate hydrate ($\text{Er}(\text{CH}_3\text{CO}_2)_3 \cdot x\text{H}_2\text{O}$, 99.9%), neodymium (III) acetate hydrate ($\text{Nd}(\text{CH}_3\text{CO}_2)_3 \cdot x\text{H}_2\text{O}$, 99.9%), oleic acid (OA, 65-88%), 1-octadecene (90%), ammonium fluoride (NH_4F , $\geq 98\%$), N-hydroxysuccinimide (NHS, 98%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, commercial grade), 1-hydroxybenzotriazole hydrate (HOBT, 97%), 5-norbornene-2-endo-acetic acid, dibenzocyclooctyne-amine (DBCO- NH_2), diethylene glycol ($\geq 99\%$), sodium citrate tribasic dihydrate ($\geq 99\%$), chloramphenicol (bioreagent grade), vancomycin hydrochloride (pharmaceutical secondary standard) were purchased from Sigma-Aldrich. 4-oxo-4(((6-(6-(pyridine-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridine-3-yl)butanoic acid (dHTz-COOH **1a**) and 2-amino-N-(6-(6-(pyridine-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridine-3-yl)acetamide (dHTz- NH_2) were purchased from Biocone Frontier Bio-chem Reagents. Methanol (HPLC grade), cyclohexane (HPLC grade) and dimethylformamide (DMF, HPLC grade) were purchased from Tedia. NaOH ($> 99\%$) was purchased from Sinopharm Chemical Reagent Co. Ltd. Toluidine blue O (for microscopy) and N,N-diisopropylethylamine (DIPEA, for peptide synthesis, $\sim 2 \text{ M}$ in 1-methyl-2-pyrrolidinone) were purchased from Fluka. N,N,N',N'-Ethylenediaminetetraacetic acid, disodium salt, dihydrate (EDTA, $\geq 99\%$) was purchased from USB Corporation. N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate

(HBTU, 99.67%) was purchased from GL Biochem (Shanghai) Ltd. 3-azido-D-alanine HCl (ADA) was purchased from Jena Bioscience, and AFDye555-DBCO was purchased from Click Chemistry Tools. KCl ($\geq 99.5\%$), KH_2PO_4 ($\geq 99.5\%$), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ($\geq 99.5\%$) were purchased from Merck KGaA. NaCl (99.9%) was purchased from VWR BDH Prolabo® Chemicals. All chemicals were used as received. Three bacterial strains were used; Van-resistant *Enterococcus faecium* (VanA genotype, ATCC 51559) and *Enterococcus faecalis* (VanB genotype, ATCC 51299), and Van-susceptible *B. subtilis* (ATCC 6051).

3.2.2 Core UCNC synthesis

$\text{Y}(\text{CH}_3\text{CO}_2)_3$ (72.89 mg), $\text{Yb}(\text{CH}_3\text{CO}_2)_3$ (50.66 mg), $\text{Er}(\text{CH}_3\text{CO}_2)_3$ (0.763 mg) and $\text{Nd}(\text{CH}_3\text{CO}_2)_3$ (1.289 mg) were diluted with methanol (15 mL) in a vial. The solution was added into a three-neck round bottom flask after 30 min sonication. Oleic acid (3 mL) and 1-octadecene (7 mL) was added into the three-neck round bottom flask. The mixture solution was heated to 100 °C and kept in vacuum to remove methanol oxygen and water. The mixture solution was further heated to 150 °C and maintained in 150 °C for 1 h under N_2 environment. The mixture solution was then cooled to room temperature. After cooling down to room temperature, NaOH (1mmol), NH_4F (1.6 mmol) and methanol (6 mL) were added into the mixture solution and the mixture solution was heated to 50 °C and kept at 50 °C under stirring for 30 min. Subsequently the mixture solution was heated to 100 °C and kept under vacuum to remove water, oxygen and methanol before bringing the temperature slowly up to 300 °C and

maintained for 2 h under N₂ atmosphere. The mixture was cooled to room temperature, washed twice with ethanol and centrifuged to retrieve the nanoparticles.

3.2.3 Core-shell UCNC synthesis

Y(CH₃CO₂)₃ (95.77mg) and Nd(CH₃CO₂)₃ (12.89 mg) were first dissolved in methanol (18 mL) in a three-neck round bottom flask, followed by the addition of a cyclohexane solution containing core nanoparticles (2 mL), 1-octadecene (7 mL) and oleic acid (3 mL). Similar procedures were conducted following in Section 3.2.2. The temperature of the mixture solution was increased to 310 °C and maintained for 3 h kept in nitrogen environment. All core and core-shell nanocrystals were characterized using TEM (JEM 1400, Jeol) and AFM (MultiMode 8, Bruker) and XRD (D8 Advance, Bruker).

3.2.4 Ligand exchange of UCNC

A mixture of sodium citrate (2 mmol) and diethylene glycol (15 mL) was first heated to 110 °C under N₂ environment for 30 min. Core-shell UCNC in cyclohexane (2 mL) and toluene (4 mL) were added into the mixture. The reaction mixture was heated to 160 °C under N₂ flow for 30 min to evaporate off the cyclohexane and toluene. The reaction mixture was further maintained at 160 °C for 3 h under N₂ environment before cooling to room temperature. The mixture was subsequently centrifuged, and the retrieved cit-capped core-shell UCNC washed three times with deionized water before dispersing in DI water (2 mL). The emission spectrum of cit-capped core-shell UCNC

was recorded by exciting the sample using an 808 nm DPSS laser (VA-I-DC-808, Beijing Viasho Technology) and the spectrum recorded using an optical spectrometer (AvaSpec-ELS2048, Avantes).

3.2.5 Linking toluidine blue O to cit-capped UCNC

Cit-capped core-shell UCNC and toluidine blue O (TB, 0.5 mg) were first added into DMF (600 μ L). 50 μ L of NHS in DMF (40 mM), 100 μ L of EDC in DMF (40 mM) and 50 μ L of HOBT in DMF (40 mM) were added into the DMF mixture of TB and cit-capped UCNC. The reaction mixture was stirred for 4 h at room temperature. Following that, the TB-UCNC were retrieved by centrifugation and washed three times with deionized water.

3.2.6 Synthesis of NaYF₄@NaYF₄ nanoparticles

Y(CH₃CO₂)₃ (106.42 mg) was diluted with methanol (15 mL) in a vial. The solution was added into a three-neck round bottom flask after 30 min sonication. Oleic acid (3 mL) and 1-octadecene (7 mL) was added into the three-neck round bottom flask. The mixture solution was heated to 100 °C and kept in vacuum to remove methanol oxygen and water. The mixture solution was further heated to 150 °C and maintained in 150 °C for 1 h under N₂ environment. The mixture solution was then cooled to room temperature. After cooling down to room temperature, NaOH (1mmol), NH₄F (1.6 mmol) and methanol (6 mL) were added into the mixture solution and the mixture solution was heated to 50 °C and kept at 50 °C under stirring for 30 min. Subsequently

the mixture solution was heated to 100 °C and kept under vacuum to remove water, oxygen and methanol before bringing the temperature slowly up to 300 °C and maintained for 2 h under N₂ atmosphere. The mixture was cooled to room temperature, washed twice with ethanol and centrifuged to retrieve the nanoparticles.

Y(CH₃CO₂)₃ (106.42mg) was first dissolved in methanol (18 mL) in a three-neck round bottom flask, followed by the addition of a cyclohexane solution containing core nanoparticles (2 mL), 1-octadecene (7 mL) and oleic acid (3 mL). Similar procedures were followed as NaYF₄ core nanoparticles synthesis. The temperature of the mixture solution was increased to 310 °C and maintained for 3 h kept in nitrogen environment. The mixture was then cooled to room temperature, washed twice with ethanol and centrifuged to retrieve the NaYF₄@NaYF₄ nanocrystals.

3.2.7 Synthesis of Nb-DBCO

DBCO-NH₂ (28 mg), 5-norbornene-2-endo-acetic acid (16 mg), HBTU (40 mg) and DIPEA (10 µL) were dissolved in DMF (500 µL) and stirred for 4 h at room temperature. Nb-DBCO was isolated by reversed-phase HPLC (Waters 2695 Separations Module) and lyophilized. NMR spectroscopy was carried out on an NMR spectrometer (Ultrashield 400 Plus, Bruker). Liquid chromatography-mass spectrometry (LCMS) was measured using a LCMS spectrometer (UltiMate 3000, Thermo Scientific). The ¹H NMR spectra of 5-norbornene-2-endo-acetic acid, DBCO and Nb-DBCO are presented in Figure 3.5.1 and 3.5.2. The LCMS spectrum of Nb-DBCO is presented in Figure 3.5.3.

The Nb-DBCO was identified by the MS peak at m/z 411.27. Calculated $[M+H]^+$: 411.52.

^1H NMR (400 MHz, DMSO- d_6) δ 7.67 – 7.56 (m, 2H), 7.54 – 7.43 (m, 3H), 7.42 – 7.38 (m, 1H), 7.38 – 7.26 (m, 2H), 6.09 (dt, $J = 5.0, 2.1$ Hz, 1H), 5.33 (t, $J = 4.8$ Hz, 1H), 5.05 (d, $J = 14.0$ Hz, 1H), 3.09 (dt, $J = 13.5, 7.0$ Hz, 2H), 2.91 (dd, $J = 13.2, 6.5$ Hz, 1H), 2.58 (d, $J = 3.9$ Hz, 1H), 2.18 (t, $J = 7.3$ Hz, 2H), 1.98 (d, $J = 6.1$ Hz, 2H), 1.24 (d, $J = 5.2$ Hz, 10H), 0.86 (s, 2H).

3.2.8 Synthesis of Van-dHTz

dHTz-NH₂ (4 mg), Van (16 mg), **HBTU (8 mg)** and **DIPEA** (10 μL) were dissolved in DMF (100 μL) and stirred for 4 h at room temperature. The Van-dHTz was purified by reversed-phase HPLC and lyophilized. The ^1H NMR spectra of dHTz-NH₂, Van and Van-dHTz are presented in Figure 3.5.4 and 3.5.5. The LCMS spectrum of Van-dHTz is presented in Figure 3.5.6.

The Van-dHTz was identified by the MS peak at m/z 871.06. Calculated $[M/2+H]^+$: 871.795.

^1H NMR (400 MHz, Deuterium Oxide) δ 8.84 – 8.68 (m, 1H), 8.60 (dt, $J = 4.7, 1.3$ Hz, 1H), 8.08 (dd, $J = 8.7, 2.5$ Hz, 1H), 8.03 – 7.88 (m, 2H), 7.75 (s, 1H), 7.65 – 7.42 (m, 2H), 7.30 (s, 1H), 7.14 (s, 1H), 6.47 (t, $J = 2.5$ Hz, 1H), 5.68 (s, 1H), 5.52 – 5.21 (m, 3H), 4.48 (s, 1H), 4.21 (s, 1H), 3.98 (s, 1H), 3.74 (s, 1H), 3.58 (s, 1H), 3.39 (s, 1H), 3.29 (s, 1H), 2.72 (d, $J = 7.9$ Hz, 2H), 2.63 (p, $J = 2.0$ Hz, 2H), 2.01 (d, $J = 12.0$ Hz, 1H), 1.38 (s, 1H), 1.13 (d, $J = 6.2$ Hz, 1H), 0.88 (dd, $J = 14.4, 6.2$ Hz, 3H).

3.2.9 ATR-FTIR measurements

OA-capped core-shell UCNC, cit-capped core-shell UCNC, and TB-UCNC were dried overnight in an oven, and separately deposited onto an ATR crystal. The ATR-FTIR spectra were measured using an FTIR spectrometer (Spectrum 100, PerkinElmer).

3.2.10 Luminescence lifetime measurements

The time-resolved luminescence lifetime decay profiles of cit-capped UCNC and TB-UCNC were recorded using a fluorescence lifetime microscope (PicoQuant, MicroTime 200).

3.2.11 UCNC quantum yield measurement

OA-capped core-shell UCNC in cyclohexane (20 mg mL⁻¹) and NaYF₄@NaYF₄ nanoparticles in cyclohexane (20 mg mL⁻¹) were first prepared. The quantum yield of the OA-capped UCNC was calculated using the equation: $Q_0 = (\text{photons emitted} / \text{photons absorbed})$, where photons emitted is the emission intensity of the OA-capped core-shell UCNC¹⁵⁰. $\text{Photons absorbed} = P[\text{reference}] - P[\text{sample}]$, where $P[\text{reference}]$ and $P[\text{sample}]$ are intensities of the excitation light after passing through the NaYF₄@NaYF₄ nanoparticle solution and OA-capped core-shell UCNC solution, respectively. The samples were excited using an 808 nm DPSS laser (VA-I-DC-808, Beijing Viasho Technology) and the light detected using a spectrometer (AvaSpec-ELS2048, Avantes).

3.2.12 Estimating the number of TB molecules linked to a cit-capped UCNC

A series of TB in PBS buffer with known concentrations (1.0, 2.0, 4.0, 8.0, 10.0 $\mu\text{g mL}^{-1}$) were prepared. Reversed-phase HPLC experiments were performed to obtain a calibration curve based on the peak area at 650 nm. 10 μL of the stock TB-UCNC solution was diluted to 1 mL and HPLC measurement was conducted to determine the mass of TB linked to the UCNC. The mass of TB was determined to be 0.541 μg . 10 μL of the same stock solution of TB-UCNC was dried in an oven to obtain the mass of TB-UCNC. The determined mass of UCNC was 89.549 μg .

The volume of a hexagonal unit cell is given by: $V_{\text{cell}} = (\sqrt{3}/2)a^2c = 0.1086 \text{ nm}^3$, where the lattice parameters for hexagonal unit cells a and c are 5.96 $\text{\AA}$ and 3.53 $\text{\AA}$, respectively¹⁵¹⁻¹⁵². Assume that the core nanoparticle is a sphere with an average diameter $d = 29.4 \text{ nm}$, and the core-shell nanocrystal is a cylinder with an average length and width of $l = 59.5 \text{ nm}$ and $w = 31.7 \text{ nm}$, respectively (see Fig. 1A and B). The volumes of the core, core-shell and shell are $V_c = (4/3)\pi(d/2)^3 = 13299 \text{ nm}^3$, $V_{cs} = \pi(w/2)^2l = 46936 \text{ nm}^3$ and $V_s = V_{cs} - V_c = 33637 \text{ nm}^3$, respectively. The number of unit cells in a core is estimated by: $n_c = V_c/V_{\text{cell}} = 122459$, and the number of unit cells in a shell is estimated by: $n_s = V_s/V_{\text{cell}} = 309733$.

Given that the atomic weight of Na $AW_{\text{Na}} = 22.989 \text{ g mol}^{-1}$, F $AW_{\text{F}} = 18.998 \text{ g mol}^{-1}$, Y $AW_{\text{Y}} = 88.905 \text{ g mol}^{-1}$, Nd $AW_{\text{Nd}} = 144.242 \text{ g mol}^{-1}$, Yb $AW_{\text{Yb}} = 173.054 \text{ g mol}^{-1}$ and Er $AW_{\text{Er}} = 167.259 \text{ g mol}^{-1}$. The atomic weight of a single hexagonal unit cell of the core ($\text{NaYF}_4:\text{Nd}^{3+}/\text{Yb}^{3+}/\text{Er}^{3+}$) is calculated by: $AW_c = (1.5 \times AW_{\text{Na}}) + (6 \times AW_{\text{F}}) + 1.5 \times (0.685 \times AW_{\text{Y}} + 0.01 \times AW_{\text{Nd}} + 0.3 \times AW_{\text{Yb}} + 0.005 \times AW_{\text{Er}}) = 308.49 \text{ g mol}^{-1}$.^{R2} The

atomic weight of a single hexagonal unit cell of the shell ($\text{NaYF}_4\text{:Nd}^{3+}$) is calculated by: $AW_s = (1.5 \times AW_{\text{Na}}) + (6 \times AW_{\text{F}}) + 1.5 \times (0.9 \times AW_{\text{Y}} + 0.1 \times AW_{\text{Nd}}) = 290.13 \text{ g mol}^{-1}$. The molecular weight of the core-shell UCNC $MW_{cs} = (n_c \times AW_c) + (n_s \times AW_s) = 1.28 \times 10^8 \text{ g mol}^{-1}$. From the determined mass of TB and UCNC, the number of TB molecules linked to a UCNC is 2528.

3.2.13 Conversion of dHTz 1a to Tz 1b

PBS buffer was prepared by adding the following to deionized water: NaCl (8 g L⁻¹), KCl (0.2 g L⁻¹), Na₂HPO₄ (1.42 g L⁻¹), KH₂PO₄ (0.24 g L⁻¹) and EDTA (0.672 g L⁻¹), and the pH adjusted to 7.4 using 1 M NaOH solution. The TB-UCNC was first washed with PBS buffer (1 mL) twice before dispersing in PBS buffer (0.5 mL) for further experiments. A stock solution of dHTz **1a** was first prepared. 0.5 mL of the stock dHTz **1a** solution was added into 0.5 mL PBS, and the absorption spectrum of dHTz **1a** was recorded using a UV-visible spectrometer (Cary 100, Varian). A mixture of dHTz **1a** (0.5 mL) and TB-UCNC in PBS buffer (0.5 mL) was stirred for 5 min under the irradiation of an 808 nm laser (4.3 W cm⁻²) (MDL-H-808nm-2.5W, Changchun New Industries Optoelectronics Technology Co., Ltd). The mixture was subsequently centrifuged at 10000 rpm for 10 min to remove TB-UCNC, and the absorption spectrum of the supernatant measured. Various amounts of TB-UCNC were used ranging from 0 to 30 mg mL⁻¹. A similar experiment was performed whereby a thin (1.5 mm) slice of chicken breast meat was placed between the bacterial solution and laser source during light exposure. In this case, the amount of TB-UCNC used was 25.0 mg mL⁻¹.

The following control experiments were also performed. Three separate solutions were prepared: (i) a PBS solution of dHTz **1a**, (ii) a PBS solution of dHTz **1a** and TB (29.4 μM), and (iii) a PBS solution of dHTz **1a** and cit-capped UCNC (25.0 mg mL^{-1}). These solutions were irradiated with 808 nm light (4.3 W cm^{-2}) for 5 min, and the absorption spectra of the solutions recorded. A fourth solution containing dHTz **1a** and TB-UCNC (25.0 mg mL^{-1}) was kept in the dark for 5 min before removing the TB-UCNC by centrifugation. The absorption spectrum of the supernatant was then recorded.

3.2.14 Conversion of Van-dHTz to Van-Tz

A Van-dHTz solution in PBS buffer with OD ~ 0.6 at 284 nm was prepared. The Van-dHTz solution was then mixed with TB-UCNC (25.0 mg mL^{-1}), and irradiated with 808 nm light (4.3 W cm^{-2}) for 5 min under continuous stirring. The solution was centrifuged to remove the TB-UCNC, and the supernatant collected for UV-vis absorption measurement.

3.2.15 Uptake of ADA by bacteria

B. subtilis, VanA and VanB bacterial cells were seeded and grown on Lysogeny broth and shaken overnight (OD₆₀₀ ~ 0.9 , 0.83 and 0.85 for *B. subtilis*, VanA and VanB). ADA (10 mM) was added to the bacterial solution and incubated at 37°C for 1 h. Subsequently, the bacterial solution was centrifuged to get bacterial pellets. The bacteria was then washed with PBS buffer (pH 7.4), re-suspended in PBS buffer and incubated for 1 h with DBCO functionalized AFDye555 (AFDye555-DBCO). The

bacteria with AFDye555 dye functionalized on the cell surface were washed with PBS buffer before deposition onto glass coverslips and visualized using a confocal microscope (LSM 800, Carl Zeiss). The excitation wavelength was 561 nm and the emission wavelength range monitored was from 565 nm to 630 nm.

3.2.16 DBCO-Nb and TB-UCNC toxicity experiments

B. subtilis, VanA and VanB bacteria were seeded and grown on Lysogeny broth and shaken overnight. ADA (10 mM) was added to the bacterial solution and incubated at 37°C for 1 h. Subsequently, the bacterial solution was centrifuged to get bacterial pellets. The bacteria was then washed with PBS buffer (pH 7.4), re-suspended in PBS buffer and incubated for 1 h with Nb-DBCO (10 mM). After washing the bacteria whose cell walls are functionalized with Nb with PBS buffer, the cells were re-suspended in Lysogeny broth and shaken for 3 h before being plated on a LB agar. The number of colony forming units (CFU) was counted after 12 h incubation at 37 °C.

To assess the toxicity of TB-UCNC, bacteria cells were seeded and grown in Lysogeny broth and shaken overnight. TB-UCNC (25.0 mg mL⁻¹) was added into the bacterial solution and irradiated with an 808 nm laser for 5 min under continuous stirring. The solution was further shaken for 3 h before plated on a LB agar. The number of CFU was counted after 12 h incubation at 37 °C.

3.2.17 MIC of Van against *B. subtilis*, VanA, VanB

B. subtilis, VanA, VanB bacterial cells were seeded and grown in Lysogeny broth and

shaken overnight. The bacterial solution was diluted 5-times with Lysogeny broth and then incubated for another 3 h. Various concentrations of Van (100 μL) were separately added into the bacterial solution (100 μL) and shaken for 3 h. 10 μL of a 100-times diluted bacterial solution was then applied onto LB agar plates and the concentration of Van that resulted in 90 % bacterial growth inhibition was taken to be the MIC value.

3.2.18 MIC of Van-dHTz against *B. subtilis*, VanA and VanB

B. subtilis, VanA, VanB bacteria cells were seeded and grown on Lysogeny broth and shaken overnight. ADA (10 mM) was added to the bacterial solution and incubated at 37°C for 1 h. Subsequently, the bacterial solution was centrifuged to get bacterial pellets. After washing, the bacteria were re-suspended in PBS buffer and incubated for 1 h with Nb-DBCO (10mM). The Nb-functionalized bacteria were recovered by centrifugation and washed using PBS buffer. Various concentrations of Van-dHTz and TB-UCNC (25.0 mg mL⁻¹) were added into the bacterial solution and irradiated with 808 nm light (4.3 W cm⁻²) for 5 min. The bacteria were recovered by centrifugation, washed using PBS buffer, re-suspended in Lysogeny broth and incubated for another 3 h before being plated on an LB agar. The CFU values and MIC were determined. For VanB, a similar experiment was performed whereby a thin (1.5 mm) slice of chicken breast meat was place between the bacterial solution and laser source during light exposure.

3.2.19 Effect of NIR light irradiation

B. subtilis, VanA, VanB bacteria cells were seeded and grown on Lysogeny broth and

shaken overnight. ADA (10 mM) was added to the bacterial solution and incubated at 37°C for 1 h. Subsequently, the bacterial solution was centrifuged to get bacterial pellets. After washing, the bacteria were re-suspended in PBS buffer and incubated for 1 h with Nb-DBCO (10 mM). The Nb-functionalized bacteria were recovered by centrifugation and washed using PBS buffer. Nb-functionalized bacteria were mixed and stirred with Van-dHTz (concentrations of Van are 0.09, 27.1, 9.6 $\mu\text{g mL}^{-1}$ for *B. subtilis*, VanA and VanB, respectively) and TB-UCNC (25.0 mg mL^{-1}) while being exposed to 808 nm light (4.3 W cm^{-2}) for 5 min. Unbound Van-dHTz/Van-Tz was removed by centrifugation, and the bacteria washed with PBS buffer before re-suspension in Lysogeny broth. The bacterial cells were incubated at 37 °C for 3 h before plated on a LB agar. In a control experiment, the above procedure was repeated in the absence of NIR light irradiation.

3.2.20 Intracellular accumulation of UDPMurNac-pp

B. subtilis, VanA, VanB bacteria cells were seeded and grown on Lysogeny broth and shaken overnight. Bacterial cell concentration was adjusted to OD₆₀₀ 0.6 and incubated with chloramphenicol (130 $\mu\text{g mL}^{-1}$) for 15 min. For each bacteria, three samples were prepared. The first was untreated bacteria. The second was bacterial solution with Van (0.11, 27.0 and 8.9 $\mu\text{g mL}^{-1}$ for *B. subtilis*, VanA and VanB, respectively) added and incubated for 1 h before removal of Van by centrifugation. The third was Nb-functionalized bacterial solution with Van-dHTz (Van concentrations of 0.11, 27.0 and 8.9 $\mu\text{g mL}^{-1}$ for *B. subtilis*, VanA and VanB, respectively) added followed by 808 nm

light (4.3 W cm^{-2}) irradiation for 5 min in the presence of TB-UCNC (25.0 mg mL^{-1}). Unbound Van-dHTz/Van-Tz was removed by centrifugation and the bacterial solution incubated for 1 h. The bacteria were washed with DI water and transferred into boiling water and treated for 30 min. The cell extract after the boiling water treatment was then retrieved by centrifugation. The supernatant's pH was adjusted to 2.0 with phosphoric acid and DI water. The UDPMurNac-pp was isolated by HPLC and identified by LCMS. The absorbance peak of UDPMurNac-pp at 260 nm was measured using an absorption spectrometer for the three bacterial strains and compared.

3.3 Results and discussion

3.3.1 Characterization of TB-UCNC

The core-shell UCNC is prepared by first synthesizing the core nanoparticle comprising of NaYF_4 matrix co-doped with Nd^{3+} (1 mol%), Yb^{3+} (30 mol%) and Er^{3+} (0.5 mol%) (*i.e.*, $\text{NaYF}_4: \text{Nd}^{3+}/\text{Yb}^{3+}/\text{Er}^{3+}$). The TEM image reveals a spherical core with an average diameter of $29.4 \pm 1.6 \text{ nm}$ (Figure 3.2A and Figure 3.3A). A layer of NaYF_4 doped with Nd^{3+} (10 mol%) shell is deposited onto the core to facilitate energy harvesting and prevent surface quenching.⁸ The slightly elongated core-shell UCNC (*i.e.*, $\text{NaYF}_4: \text{Nd}^{3+}/\text{Yb}^{3+}/\text{Er}^{3+} @ \text{NaYF}_4: \text{Nd}^{3+}$) exhibits an average length and width of $59.5 \pm 1.6 \text{ nm}$ and $32.8 \pm 1.7 \text{ nm}$, respectively (Figure 3.2B, 3.2C and Figure 3.3B). The average height of the UCNC, measured from atomic force microscopy (AFM), is $30.5 \pm 1.4 \text{ nm}$ (Figure 3.2D and Figure

3.3C). Given that the width and height are approximately similar, we assume that the core-shell UCNC adopts a cylinder-like morphology with a diameter $\sim 31.7 \pm 2.2$ nm (*i.e.*, average of the width and height). This means that along the curved surface of the core-shell UCNC, a large portion of the core is only thinly coated with a layer of shell (< 2.5 nm thickness). The X-ray diffraction (XRD) patterns of the core nanoparticles and core-shell nanocrystals suggest that they are of hexagonal phase (Figure 3.1).

Figure 3.1 XRD patterns of core NaYF₄: Nd³⁺/Yb³⁺/Er³⁺ nanoparticle (black) and core-shell NaYF₄:Nd³⁺/Yb³⁺/Er³⁺@NaYF₄:Nd³⁺ nanocrystals (red). The peaks for both the core nanoparticles and core-shell nanocrystals are well indexed to the peaks of standard hexagonal phase β -NaYF₄ (JCPDS No. 16-0334).

Hydrophilic UCNC are obtained by replacing the oleic acid (OA) ligands on the as-prepared core-shell UCNC with citrate (cit) *via* a ligand exchange reaction.^{30,31} The morphology of the cit-capped UCNC remains relatively

invariant after ligand exchange with an average length and width of 62.7 ± 2.7 nm and 32.8 ± 2.4 nm, respectively (Figure 3.4B and C). The attenuated total reflection-Fourier-transform infrared (ATR-FTIR) spectrum of the OA-capped UCNC (Figure 3.3D) shows characteristic bands associated with the OA's methylene group stretching (2920 and 2851 cm^{-1}) and carboxyl group stretching (1558 and 1465 cm^{-1}) vibrations. The ATR-FTIR spectrum of the cit-capped UCNC shows a 1638 cm^{-1} peak corresponding to the asymmetric stretching mode of the citrate carboxyl groups; indicating successful ligand exchange. The broad band at 3381 cm^{-1} is ascribed to O-H stretching, possibly from unremoved water from the sample.

Figure 3.2 Histograms of the size distribution of the as-prepared core upconversion nanoparticles and core-shell upconversion nanocrystals. (A) Histogram of the diameter of the as-prepared core. The Gaussian fit gives an average diameter of 29.4 ± 1.6 nm. (B) Histogram of the length of the as-prepared core-shell UCNC. The Gaussian fit gives an average length of 59.5 ± 1.6 nm. (C) Histogram of the width of the as-prepared core-shell UPNC. The Gaussian fit gives an average width of 32.8 ± 1.7 nm. (D) Histogram of the height of the as-prepared core-shell UCNC. The Gaussian fit gives an average height of 30.5 ± 1.4 nm. (A)-(C) are based on 100 nanocrystals, and (D) is based on 12 nanocrystals.

Figure 3.3 TEM image of OA-capped core (A) and core-shell (B) UCNC. (C) AFM image of a group of OA-capped core-shell UCNC arranged next to each other on the substrate. The inset shows the cross-section plot of a UCNC along the dotted line with the blue cross. (D) FTIR spectra of OA-capped UCNC (black) and cit-capped UCNC after ligand exchange (red).

Figure 3.4 (A) TEM image of cit-capped core-shell UCNC after ligand exchange. (B) Histogram of the length of the cit-capped core-shell UCNC. The Gaussian fit gives an average length of 62.7 ± 2.7 nm. C) Histogram of the width of the cit-capped core-shell UCNC. The Gaussian fit gives an average width of 32.8 ± 2.4 nm.

Figure 3.5 ATR-FTIR spectra of TB-UCNC (red) and TB (black). The intense 1600 cm^{-1} peak observed for both TB-UCNC and TB arises from the dye's aromatic rings vibrational modes. This peak is not present in the ATR-FTIR spectrum of cit-capped UCNC (Figure 3.3D).

The toluidine blue O (TB) photocatalyst is covalently attached to the citrate ligands on the nanocrystal surface through amide bond formation between the amine group on TB and carboxylic acid group on citrate. The ATR-FTIR spectrum of the UCNC whose surface is decorated with TB (*i.e.*, TB-UCNC) reveals a peak at 1600 cm^{-1} which is attributed to the vibrational mode of the dye's aromatic rings (Figure 3.5). The upconversion luminescence spectrum (UCL) of the cit-capped UCNC in water recorded after $\lambda_{\text{ex}} = 808 \text{ nm}$ light excitation reveals emission peaks arising from Er^{3+} transitions at *ca.* 519 nm ($^2\text{H}_{11/2} \rightarrow ^4\text{I}_{15/2}$), 538 nm ($^4\text{S}_{3/2} \rightarrow ^4\text{I}_{15/2}$) and 653 nm ($^4\text{F}_{9/2} \rightarrow ^4\text{I}_{15/2}$) (Figure 3.6A). Significant spectral overlap between the absorption spectrum of TB and UCL spectrum of cit-capped UCNC suggests feasible resonance energy transfer (ET)

between TB and UCNC when the latter is excited by 808 nm light (Figure 3.6A). Luminescence quenching of the photo-excited UCNC by ET to TB is supported by measuring the time-resolved emission lifetime decay profiles of the UCNC in the absence and presence of bound TB. As shown in Figure 3.6 B, the luminescence lifetime of the cit-capped UCNC is $\tau_A = 502 \mu s$ which is shortened to $\tau_{AA} = 315 \mu s$ when TB is linked to the nanocrystals. The energy transfer efficiency $E = 1 - \tau_{DA}/\tau_D$ is calculated to be 37.3 %.

Based on Förster theory, the ET efficiency is theoretically defined as:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

where r is the distance between the energy donor (D) and acceptor (A) and the Förster critical distance R_0 is defined as:

$$R_0^6 = \frac{9Q_0\kappa^2J(\ln 10)}{128\pi^5n^4N_A}$$

In the above equation Q_0 is the emission quantum yield of D in the absence of A , κ^2 is the dipole orientation factor, J is the spectral overlap integral and n is the refractive index of the medium. For the system studied, the energy transfer takes place from a single UCNC donor to several TB acceptors conjugated onto the surface of the nanocrystal. A modified expression to estimate the ET efficiency from one donor to N acceptors with a similar D - A separation (r) is given by:

$$E = \frac{NR_0^6}{NR_0^6 + r^6}$$

In this case, a Förster critical distance $R_0 \approx 4.2 \text{ Å}$ is determined using the PhotochemCAD program for $Q_0 = 1.58 \times 10^{-7}$, and assuming $\kappa^2 = 2/3$ for freely rotating TB molecules bound to the surface of the UCNC. The number of TB molecules attached onto a UCNC is estimated to be $N \approx 2528$ (see section 3.2.12). An energy transfer efficiency of 37.3 % is achieved when the separation between all the TB and UCNC is $r \approx 1.7 \text{ nm}$. For our system, even though TB molecules attached at or close to the base surfaces of the nanocrystals undergo ineffective ET with the Er^{3+} ions in the core due to their long spatial separation, it is reasonable to propose that TB along the curved surface of the nanocrystals may be sufficiently close to the emitting Er^{3+} ions to undergo efficient Förster energy transfer.

Figure 3.6 (A) Absorption spectrum of toluidine blue O (red) and UCL spectrum of cit-capped UCNC (blue) in water. The UCL is recorded by exciting the sample using 808 nm light. (B) Luminescence lifetime decay curves of cit-capped UCNC (black data points) and TB-UCNC (red data points) in water. The samples are excited using 808 nm light and upconversion emission from 650 - 720 nm monitored. The mono-exponential decay fits, given as solid curves, yield emission lifetimes of 502 and 315 μ s for cit-capped UCNC and TB-UCNC, respectively.

3.3.2 Conversion of **1a** to **1b**

To assess the effectiveness of TB in catalysing the oxidation of dHTz-COOH **1a** to Tz-COOH **1b** (Scheme 3.1A), a PBS solution (pH 7.4) of **1a** and TB is irradiated with visible light (*i.e.*, 633 nm). Before light exposure, an absorption peak at 292 nm corresponding to the absorption of **1a** is observed (Figure 3.8A). A new peak at 323 nm, attributed to the absorption of **1b**, and concomitant

disappearance of the 292 nm peak are seen when TB is directly excited by 633 nm light for 5 min (0.62 W cm^{-2}). Previous studies have shown that reactive oxygen species such as singlet oxygen $^1\text{O}_2$ generated from excited photosensitizers (*e.g.*, methylene blue and rose bengal) are unable to oxidize dHTz, and the conversion of **1a** to **1b** is therefore due to the photocatalytic activity of TB.

The absorption spectrum of **1a** in PBS buffer remains unchanged when exposed to 808 nm light (4.3 W cm^{-2}) for 5 min (spectrum a in Figure 3.7A). This means that **1a** is not oxidized to **1b** when irradiated with NIR light. Even in the presence of TB, NIR light is unable to induce **1a** to **1b** conversion (Figure 3.8B) since the dye molecule does not absorb 808 nm light (Figure 3.6A). When TB is replaced with TB-UCNC, the absorption spectrum of **1a** again displays no change when the solution is kept in the dark for 5 min before measurement (spectrum b in Figure 3.7A). On the other hand, when a mixture of **1a** and TB-UCNC is irradiated with 808 nm light (4.3 W cm^{-2}) for 5 min, the reduction in the absorbance at 292 nm from **1a** and concomitant increase in the absorbance at 323 nm from **1b** are clearly observed (spectra c to f in Figure 3.7A). The extent of **1a** to **1b** conversion is enhanced when a larger concentration of TB-UCNC is used. In particular, the peak intensity of **1b** does not show further increase when the TB-UCNC concentration used is $> 25.0 \text{ mg mL}^{-1}$. Furthermore, no obvious detection of **1b** absorbance is seen when a mixture of **1a** and cit-capped UCNC (*i.e.*, without TB linked to nanocrystal surface) is irradiated with 808 nm light for

5 min (spectrum g in Figure 3.7A). We have therefore shown that in the absence of either TB or UCNC, the oxidation of **1a** to **1b** does not take place. We propose that the TB molecules attached onto the UCNC are excited by upconverted energy transfer which induces them to catalyse the conversion of **1a** to **1b**.

Figure 3.7 (A) UV-vis absorption spectra of **1a** in PBS buffer after 808 nm light exposure for 5 min (a), **1a** and TB-UCNC (25.0 mg mL⁻¹) kept in the dark for 5 min (b), **1a** in the presence of various concentrations of TB-UCNC (2.8 (c), 13.9 (d), 25.0 (e), and 30.5 (f) mg mL⁻¹) after 808 nm light exposure for 5 min, and **1a** and cit-capped UCNC (25.0 mg mL⁻¹) after 808 nm light exposure for 5 min (g). The slight kink observed at 350 nm is due to the lamp change in the absorption spectrometer. (B) UV-vis absorption spectra of Van-dHTz (red), and Van-dHTz and TB-UCNC (25.0 mg mL⁻¹) after 808 nm light irradiation for 5 min. The solvent is PBS buffer. For both (A) and (B), UCNC were removed by centrifugation before measurements.

Figure 3.8 (A) UV-vis absorption spectra of **1a** before (black) and after (red) exposure to 633 nm light (Melles Griot HeNe laser) for 5 min in the presence of TB (29.4 μ M). (B) UV-vis absorption spectra of **1a** before (black) and after (red) exposure to 808 nm light for 5 min in the presence of TB.

3.3.3 Upconverted-light assisted bioorthogonal conjugation of Van to bacteria

The bioorthogonal conjugation of Van to *B. subtilis*, VanA and VanB is carried out by first labelling the bacteria with an azide group. This is achieved by incorporating the D-amino acid derivative azido-D-Alanine (ADA) into the peptidoglycan (PG) cell wall. To demonstrate that a functional material (*e.g.*, dye molecule) can be readily conjugated to ADA-treated bacteria *via* click chemistry, a dye molecule functionalized with a dibenzocyclooctyne (DBCO) group is used (AFDye555-DBCO). Figure 3.9 shows distinct fluorescence images of ADA-treated bacteria after reacting with AFDye555-DBCO whereas no clear fluorescence images are seen in the case of non-treated bacteria. This shows the

efficient uptake of ADA by the bacteria followed by an azide-alkyne cycloaddition reaction that results in the conjugation of the dye to bacterial surfaces.

Equipped with the knowledge that ADA-treated bacteria can be easily functionalized with suitable molecules, two conjugate systems are prepared to enable on-demand covalent bonding of Van to ADA-treated bacteria using NIR light activation. In this case, we prepared a conjugate norbornene-DBCO (*i.e.*, Nb-DBCO, Scheme 3.1A), and a system consisting of a Van covalently linked to a dHTz *via* the C-terminal carboxylic acid group of the antibiotic (*i.e.*, Van-dHTz, Scheme 3.1A). The absorption spectrum Van-dHTz are given in Figure 3.7B. The spectrum of Van-dHTz is a sum of the absorption of Van and dHTz, and displays a peak at 284 nm. Upon 808 nm light (4.3 W cm^{-2}) irradiation in the presence of TB-UCNC (25.0 mg mL^{-1}) for 5 min, a new peak at 311 nm from the Tz derivative (*i.e.*, Van-Tz) is observed with a concomitant decrease in the 284 nm peak (Figure 3.7B). This demonstrates the (TB-UCNC)-aided oxidative conversion of Van-dHTz to Van-Tz by NIR light excitation.

Figure 3.9 (A) Chemical structure of AFDye555-DBCO (B) Confocal fluorescence microscopy images (dark field and merged dark field and bright field images) of azido-D-Alanine (ADA)-treated *B. subtilis* (top panels) and non-treated bacteria (bottom panels) mixed with AFDye555-DBCO. (C) Confocal fluorescence microscopy images (dark field and merged dark field and bright field images) of ADA-treated VanA (top panels) and non-treated bacteria (bottom panels) mixed with AFDye555-DBCO. (D) Confocal fluorescence microscopy images (dark field and merged dark field and bright field images) of ADA-treated VanB (top panels) and non-treated bacteria (bottom panels) mixed with AFDye555-DBCO. (λ_{ex} = 561 nm, λ_{em} = 565-630 nm)

The toxicity of TB-UCNC and norbonene conjugated to cell walls is assessed next. The high viability of *B. subtilis*, VanA and VanB after exposing a mixture

of bacteria and TB-UCNC to 808 nm light (4.3 W cm^{-2}) for 5 min, followed by overnight incubation suggests that TB-UCNC is non-toxic against bacteria under this condition (Figure 3.10). Any ROS produced by the photo-excited TB during light irradiation is also insufficient to kill the cells. Furthermore, the number of colony forming unit (CFU) of Nb-functionalized *B. subtilis*, VanA and VanB, prepared by reacting ADA-treated cells with Nb-DBCO, remains relatively unchanged when compared to the corresponding non-Nb functionalized cells (Figure 3.10), implying the non-toxic nature of Nb.

Figure 3.10 Bacterial colonies of *B. subtilis* (first column), VanA (second column) and VanB (third column) on agar plates for untreated bacteria (top row), bacteria treated with ADA to form Nb-functionalized bacteria (middle row), and bacteria exposed to TB-UCNC and 808 nm light irradiation (5 min). The CFU values are presented above each plate.

The antimicrobial activity of Van conjugated to the bacterial cell wall is examined by determining the minimum inhibitory concentration (MIC). The MIC values of *B. subtilis*, VanA and VanB are determined to be 0.45 (0.31), 167.0 (115.2) and 61.0 (42.1) $\mu\text{g mL}^{-1}$ (μM), respectively; consistent with previously reported values (Table 3.1 and Figure 3.11). The MIC of Van-dHTz is determined by irradiating a mixture of Nb-functionalized bacteria, Van-dHTz and TB-UCNC (25.0 mg mL^{-1}) with 808 nm light for 5 min, followed by the removal of unbound Van-dHTz/Van-Tz (Scheme 3.1A). In this case, the MIC values, based on the concentration of Van, are significantly reduced by 6- to 7-fold to 0.11 ± 0.02 (0.075 ± 0.012), 27.0 ± 0.7 (18.6 ± 0.5) and 8.9 ± 0.9 (6.1 ± 0.6) $\mu\text{g mL}^{-1}$ (μM) for *B. subtilis*, VanA and VanB, respectively (Table 3.1 and Figure 3.12); comparable to the MIC of Van dimers that exhibit high affinity to Van resistant Enterococci.

Figure 3.11 Bacteria colonies of *B. subtilis* (A), VanA (C) and VanB (E) on agar plates after treatment with various concentrations of Van. Relative bacterial growth of *B. subtilis* (B), VanA (D) and VanB (F) after treatment with various concentrations of Van with respect to the growth in the absence of Van. The data points were fit to a single-exponential decay function, and the minimum concentrations of Van needed to give 90% growth inhibition (*i.e.*, MIC) are 0.45 (0.31), 167.0 (115.2) and 61.0 (42.1) $\mu\text{g mL}^{-1}$ (μM) for *B. subtilis*, VanA and VanB, respectively.

Figure 3.12 Bacteria colonies of *B. subtilis* (A), VanA (C) and VanB (E) on agar plates after treatment with various concentrations of Van-dHTz and 808 nm light irradiation (5 min). Relative bacterial growth of *B. subtilis* (B), VanA (D) and VanB (F) after treatment with various concentrations of Van-dHTz with respect to the growth in the absence of Van. The data points were fit to a single-exponential decay function, and the MIC value taken to be the minimum concentrations of Van needed to give 90 % growth inhibition. The MIC values reported in Table 1 are based on the concentration of Van.

Table 3.1 Antibacterial activity of Van and Van-dHTz.

	Van ($\mu\text{g mL}^{-1}/\mu\text{M}$)	Van-dHTz + TB- UCNC + 808 nm light ($\mu\text{g mL}^{-1}/\mu\text{M}$)
<i>B. subtilis</i>	0.45/0.31	0.11/0.075
VanA	167.0/115.2	27.0/18.6
VanB	61.0/42.1	8.9/6.1

The effect of NIR light excitation is examined next. Nb-functionalized *B. subtilis*, VanA and VanB are separately mixed with TB-UCNC and Van-dHTz with concentrations equivalent to the MIC obtained with NIR light activation (*e.g.*, 0.09, 27.1 and 9.6 mg mL^{-1} for *B. subtilis*, VanA and VanB, respectively). After a reaction time of 5 min in the dark, unbound Van-dHTz/Van-Tz is removed and the CFU determined. Clearly, in the absence of NIR light exposure, the CFU of *B. subtilis*, VanA and VanB are not drastically changed when compared to healthy cells in the absence of Van (Figure 3.13). This is in sharp contrast to the CFU obtained when the mixture is exposed to NIR light for 5 min prior to the removal of unbound Van-dHTz/Van-Tz. In this case, the cell viability is low and the measured CFU is drastically reduced; the CFUs are decreased by *ca.* 90% compared to healthy cells (Figure 3.13). Therefore, we propose that during NIR light excitation, TB-UCNC photocatalyzes the oxidation of the dHTz

moiety in Van-dHTz to Tz. The resulting Van-Tz then undergoes an IEDDA reaction with the Nb molecules pre-attached on the bacteria; leading to the conjugation of the antibiotic to the bacterial cell walls (Scheme 3.1B). In the absence of NIR light irradiation, the bioorthogonal conjugation reaction does not take place due to a lack of excited TB needed to induce the photocatalytic reaction.

Figure 3.13 Bacterial colonies of *B. subtilis* (first column), VanA (second column) and VanB (third column) on agar plates for untreated bacteria (first row), and bacteria exposed to Van-dHTz without (second row) and with (third row) 808 nm light irradiation (5 min). The concentration of Van-dHTz used are 0.09, 27.1 and 9.6 $\mu\text{g mL}^{-1}$ for *B. subtilis*, VanA and VanB, respectively. The CFU values are presented above each plate.

Uridine diphosphate-activated *N*-acetylmuramyl-pentadespsipeptide (UDPMurNAc-pp, C₄₀H₆₄N₈O₂₇P₂), a cell wall precursor, accumulates in the cytoplasm when the biosynthesis of cell-walls is inhibited.^{25,42,43} In order to examine whether Van bound to cell walls after NIR light activation interferes with peptidoglycan synthesis, we determined the accumulation of UDPMurNAc-pp in both drug-treated and untreated cells. The amounts of accumulated UDPMurNAc-pp for untreated bacteria, bacteria treated with Van, and bacteria with Van conjugated on to the cell surface after 808 nm light irradiation (5 min) in the presence of TB-UCNC (25.0 mg mL⁻¹) are compared using UV-vis absorption spectrometry. The concentrations of Van and Van-dHTz (in terms of Van concentration) used for each bacteria correspond to the respective MIC under NIR light activation (*i.e.*, MIC reported in the third column of Table 3.1). UDPMurNAc-pp is first isolated using high-performance liquid chromatography (HPLC) (Figure 3.14) and confirmed by liquid chromatography-mass spectrometry (LCMS). In this case, the molecular mass of UDPMurNAc-pp is determined to be 1149.46 Da from peaks at m/z 1150.46 and 576.64 Da that are assigned to $[M + H]^+$ and $[M + 2H]^{2+}$, respectively (Figure 3.15A); in agreement with the calculated m/z 1149.94 Da. As shown in Figure.3.15, the absorption peaks of UDPMurNAc-pp at ~ 260 nm for *B. subtilis*, VanA and VanB are the most intense when Van is covalently attached to the cell wall upon NIR light activation in the presence of TB-UCNC. Similar to the mode of action against Van-susceptible bacteria, the antibiotic is able to significantly inhibit the

biosynthesis of the cell walls of Van-resistant bacteria as a result of enhanced binding of the drug to cell walls.

Figure 3.14 HPLC separation of UDPMurNAC-pp from *B. subtilis* (A), VanA (B), VanB (C) monitored at 260 nm. The Nb-functionalized bacteria are mixed with TB-UCNC and irradiated with 808 nm light for 5 min. The peak eluted at ~ 40 min (*) corresponds to UDPMurNAC-pp identified by LCMS.

Figure 3.15 (A) Identification of UDPMurNAc-pp, separated using HPLC, by LCMS.

The absorption spectra of UDPMurNAc-pp with $\lambda_{\text{max}} = 260$ nm for *B. subtilis* (B), VanA (C) and VanB (D). The concentrations of Van and Van-dHTz (in terms of Van concentration) used are 0.11, 27.0 and 8.9 $\mu\text{g mL}^{-1}$ for *B. subtilis*, VanA and VanB, respectively. Untreated bacteria (black) show the least amount of UDPMurNAc-pp followed by bacteria treated with Van (blue). The highest amount of UDPMurNAc-pp is observed for bacteria with bound Van after NIR light activation in the presence of TB-UCNC (red).

We next examined if the 808 nm light can activate bioorthogonal conjugation through a thin slice (1.5 mm) of chicken breast meat. The conversion of dHTz **1a** to Tz **1b** is successfully implemented by observing the disappearance of the absorption band of dHTz **1a** at 292 nm and appearance of the 323 nm band arising from Tz **1b** when a

mixture of dHTz **1a** and TB-UCNC (25.0 mg mL^{-1}) is exposed to NIR light for 5 min with the slice of meat placed between the reaction vessel and light source (Figure 3.16). In addition, the conjugation of Van-dHTz to Nb-functionalized VanB bacteria is not inhibited when a thin slice of meat is placed before the reaction vessel (Figure 3.17). The MIC ($= 11.2 \text{ } \mu\text{g mL}^{-1}$) determined is similar to that reported in Table 1 for VanB. The 808 nm light is therefore neither significantly scattered nor absorbed by the slice of meat, and is able to penetrate through it.

Figure 3.16 UV-vis absorption of dHTz **1a** (black), and **1a** after 808 nm light (4.3 W cm^{-2}) exposure for 5 min in the presence of TB-UCNC (25.0 mg mL^{-1}) with (red) and without (blue) a thin slice of chicken breast meat placed between the reactor and light source.

Figure 3.17 (A) VanB bacterial colonies on agar plate after treatment with various concentrations of Van-dHTz in the presence of TB-UCNC (25 mg mL^{-1}) and 808 nm light irradiation through a 1.5 mm thick chicken breast for 5 min. (B) Relative bacterial growth after treatment with various concentrations of Van-dHTz with respect to growth in the absence of Van-dHTz. The data points were fit to a single-exponential decay function, and the MIC value taken to be the minimum concentrations of Van needed to give 90% growth inhibition.

3.4 Conclusion

In conclusion, an on-demand and efficient strategy of bio-conjugating Van to drug-resisitant bacteria VanA and VanB is presented. NIR light is used to trigger energy transfer of upconverted photons from an UCNC to TB dyes. The excited TB subsequently photo-catalyse the conversion of Van-dHTz to Van-Tz which undergoes IEDDA reaction with strained norbornene molecules attached onto the surfaces of bacteria. The enhanced binding of the antibiotic to bacterial cell walls increases the efficacy of the drug, and renders the MIC to be reduced by 6- to 7-fold for both VanA and VanB as compared to using neat Van. The killing mechanism of the covalently attached Van is *via* the inhibition of the biosynthesis of cell walls.

3.5 Annex

Figure 3.5.1 ^1H NMR spectra of Nb, DBCO and Nb-DBCO

Figure 3.5.2 ^1H NMR spectrum of Nb-DBCO

Figure 3.5.3 LCMS spectrum of Nb-DBCO.

Figure 3.5.4 ^1H NMR spectra of dHTz-NH₂, Van and Van-dHTz.

Figure 3.5.5 ^1H NMR spectrum of Van-dHTz.

Figure 3.5.6 LCMS spectrum of Van-dHTz.

Chapter 4 Enhanced Photothermal Activity Based on Direct Conjugation of Photothermal Molecule onto Bacteria

Abstract

Photothermal therapy (PTT) has been widely used to treat bacterial infections as it can avoid the drawbacks of traditional antibiotics arising from antibiotic resistance. PTT can efficiently transform light to heat, causing bacterial cell death. ATTO740 is an organic fluorescent molecule and can be used as a photothermal agent. In this study, an efficient method of bio-conjugating ATTO740 to bacteria is proposed using near-infrared (NIR)-light activation. A Nd^{3+} -sensitized upconversion nanocrystal (UCNC) decorated with toluidine blue O (TB) on its surface undergoes upconverted energy transfer from the UCNC to TB when excited by 808 nm light. The photoexcited TB then catalyses the conversion of the dihydrotetrazine (dHTz) moiety in a ATTO740-dHTz conjugate system to tetrazine which undergoes efficient inverse electron demand Diels-Alder reaction with prior attached norbornene molecules on bacterial cell walls. The enhanced affinity of ATTO740 to bacteria by covalent bonding improves photothermal activity. Bacterial mortality increases ~20% as compared to neat ATTO740.

4.1 Introduction

Controlled conjugation or release of biomolecules plays critical roles in various biological applications¹⁵³. Ultraviolet (UV)-visible light has been extensively used in photocatalysis to induce biochemical conjugation reactions¹²²⁻¹²³. Meanwhile, the utilization of UV light is limited due to its toxicity such as DNA damage and protein degradation, and poor penetration depth¹⁵⁴⁻¹⁵⁵. Some nanomaterials such as upconversion nanoparticles have the potential to convert near-infrared (NIR) light to UV-visible light¹⁵⁶. Several biological systems consisting of the photocatalyst grafted onto the surface of UCNCs have been fabricated to catalyze reactions *via* NIR light¹³⁹.

Photothermal therapy (PTT) has shown great promise over several other therapies such as surgery, chemotherapy and radiotherapy. PTT can be more efficient to eradicate microorganisms due to its local hyperthermia mechanism¹⁵⁷. Photothermal agents (PTAs) are the most critical element in PTT. Potential PTAs such as gold nanomaterials, carbon quantum dots and organic fluorescence molecules are widely used in various applications of PTT. ATTO740 is an organic dye molecule, which can generate heat through irradiation of 760 nm light. Herein, ATTO740 is used as a PTA to trigger antimicrobial photothermal activity.

In this study, an efficient method of bio-conjugating ATTO740 dye molecules to bacteria is proposed using near-infrared (NIR)-light activation. A Nd³⁺-sensitized upconversion nanocrystal (UCNC) decorated with toluidine blue O (TB) on its surface undergoes upconverted energy transfer from the UCNC to TB when excited by 808 nm

light. The photoexcited TB then catalyzes the conversion of the dihydrotetrazine (dHTz) moiety in an ATTO740-dHTz conjugating system to tetrazine (Tz) which undergoes efficient inverse electron demand Diels-Alder reaction with prior attached norbornene molecules on bacterial cell walls. The enhanced affinity of ATTO740 to bacteria by covalent bonding improves the photothermal activity of the photothermal agent ATTO740 against *B. subtilis*, VanA and VanB.

4.2 Experimental section

4.2.1 Materials

Yttrium (III) acetate hydrate ($\text{Y}(\text{CH}_3\text{CO}_2)_3 \cdot x\text{H}_2\text{O}$, 99.9%), ytterbium (III) acetate hydrate ($\text{Yb}(\text{CH}_3\text{CO}_2)_3 \cdot x\text{H}_2\text{O}$, 99.9%), erbium (III) acetate hydrate ($\text{Er}(\text{CH}_3\text{CO}_2)_3 \cdot x\text{H}_2\text{O}$, 99.9%), neodymium (III) acetate hydrate ($\text{Nd}(\text{CH}_3\text{CO}_2)_3 \cdot x\text{H}_2\text{O}$, 99.9%), oleic acid (OA, 65-88%), 1-octadecene (90%), ammonium fluoride (NH_4F , $\geq 98\%$), N-hydroxysuccinimide (NHS, 98%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, commercial grade), 1-hydroxybenzotriazole hydrate (HOBT, 97%), 5-norbornene-2-endo-acetic acid (Nb), dibenzocyclooctyne-amine (DBCO- NH_2), diethylene glycol ($\geq 99\%$), sodium citrate tribasic dihydrate ($\geq 99\%$) were purchased from Sigma-Aldrich. dHTz- NH_2 were purchased from Biocone Frontier Bio-chem Reagents. Methanol (HPLC grade), cyclohexane (HPLC grade) and dimethylformamide (DMF, HPLC grade) dimethyl sulfoxide (DMSO, HPLC grade) were purchased from Tedia. NaOH ($> 99\%$) was purchased from Sinopharm Chemical

Reagent Co. Ltd. Toluidine Blue O (for microscopy) and *N,N*-diisopropylethylamine (DIPEA, for peptide synthesis, ~2 M in 1-methyl-2-pyrrolidinone) were purchased from Fluka. ATTO740 NHS-ester was purchased from ATTO-TEC GmbH. *N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 99.67%) was purchased from GL Biochem (Shanghai) Ltd. 3-azido-D-alanine HCl (ADA) was purchased from Jena Bioscience. All chemicals were used as received. Three bacterial strains were used; Van-resistant *Enterococcus faecium* (VanA genotype, ATCC 51559) and *Enterococcus faecalis* (VanB genotype, ATCC 51299), and Van-susceptible *B. subtilis* (ATCC 6051).

4.2.2 The synthesis of ATTO740-dHTz

dHTz-NH₂ (0.5 mg), ATTO740 (1 mg), HBTU (2 mg) and DIPEA (5 µL) were dissolved in DMF (100 µL) and stirred for 4 h at room temperature. The ATTO740-dHTz was purified by reverse HPLC and lyophilized. The LCMS spectrum of ATTO740-dHTz is presented in Figure 4.1.

The ATTO740-dHTz was identified by the MS peak at *m/z* 760.57. Calculated [M+H]⁺: 760.61.

Figure 4.1 LCMS spectrum of ATTO740-dHTz

4.2.3 Determining photothermal conversion efficiency

An ATTO740 solution in DMSO was prepared and its absorbance at 760 nm was measured. The light-to-heat conversion efficiency of ATTO740 in DMSO (200 mL) in a quartz cuvette (optical path ~ 0.5 cm) was measured by using a 760 nm laser at 1.23 W. The temperature change was recorded every 5 seconds within 600 seconds by a FLIR E5 thermal imaging camera (FLIR system, Inc.).

4.2.4 Determining the optimal concentration of ATTO740

0.01, 0.1 and 1 mg mL⁻¹ ATTO740 solution in DMSO (200 mL) were prepared. These three solutions were irradiated with a 760 nm laser (1.23 W, Changchun New Industries Optoelectronics Tech. Co., Ltd) for 5 min, respectively. The temperature rise was recorded every 5 seconds by a thermal imaging camera.

4.2.5 Antibacterial photothermal activity of conjugated ATTO740 against bacteria

B. subtilis, VanA, VanB bacteria cells were seeded and grown on Lysogeny broth and shaken overnight. ADA (10 mM) was added to the bacterial solution and incubated at 37°C for 1 h to allow for the metabolic uptake of ADA onto bacterial peptidoglycan. Subsequently, the bacteria were recovered by centrifugation and washed in PBS buffer. After washing, the bacteria were re-suspended in PBS buffer and incubated for 1 h with Nb-DBCO (10mM). The Nb-functionalized bacteria were recovered by centrifugation and washed using PBS buffer. ATTO740-dHTz (the concentration of ATTO740 was 0.1 mg mL⁻¹) and TB-UCNC (25.0 mg mL⁻¹) were added into the bacterial solution and

irradiated with 808 nm light (4.3 W cm^{-2}) for 5 min. The bacteria were recovered by centrifugation, washed using PBS buffer, re-suspended in Lysogeny broth and irradiated with 760 nm light (30 W cm^{-2}) for 15 min before being plated on a LB agar. The CFU values were determined after 12 h incubation.

4.2.6 Antibacterial photothermal activity of mixed ATTO740 against bacteria

B. subtilis, VanA, VanB bacteria cells were seeded and grown on Lysogeny broth and shaken overnight. The bacteria were suspended in PBS buffer after centrifugation. ATTO740 (0.1 mg mL^{-1}) mixed with bacteria solution were irradiated with 760 nm light (30 W cm^{-2}) for 15 min before being plated on a LB agar. The CFU values were determined after 12 h incubation.

4.2.7 The confocal imaging of bacteria conjugated with ATTO740

B. subtilis, VanA, VanB bacteria cells were seeded and grown on Lysogeny broth and shaken overnight. ADA (10 mM) was added to the bacterial solution and incubated at 37°C for 1 h to allow for the metabolic uptake of ADA onto bacterial peptidoglycan. Subsequently, the bacteria were recovered by centrifugation and washed in PBS buffer. After washing, the bacteria were re-suspended in PBS buffer and incubated for 1 h with Nb-DBCO (10mM). The Nb-functionalized bacteria were recovered by centrifugation and washed using PBS buffer. ATTO740-dHTz (the concentration of ATTO740 was 0.1 mg mL^{-1}) and TB-UCNC (25.0 mg mL^{-1}) were added into the bacterial solution and irradiated with 808 nm light (4.3 W cm^{-2}) for 5 min. The bacteria were recovered by

centrifugation and resuspended in PBS buffer before being plated in a glass slide. The bacteria conjugated with ATTO740 were excited by 740 nm and the emission imaging was captured by a confocal microscope (Nikon).

4.3 Results and discussion

Scheme 4.1 Scheme illustrating the conjugation of Van-dHTz to Nb-functionalized bacterial cell wall in the presence of TB-UCNC and 808 nm light excitation and the heat generation process with exposure to 760 nm light.

4.3.1 Calculating photothermal conversion efficiency of ATTO740

The light-to-heat conversion efficiency of ATTO at 760 nm is determined by the following equation¹⁵⁸: $\eta = \frac{hS(T_{\max} - T_{\text{surr}}) - Q_{\text{dis}}}{I_0 (1 - 10^{-A_{760}})}$, where η is the photothermal conversion efficiency; h is the heat transfer coefficient; S is the surface area of the container; $T_{\max}$ is the maximal temperature during irradiation by 760 nm light; T_{surr} is the surrounding ambient temperature; Q_{dis} is the energy input due to the solvent and container; I_0 is the power of 760 nm laser and A_{760} is the absorbance of ATTO740 solution at 760 nm.

In order to obtain the value of hS , a dimensionless driving force temperature θ is defined as $\theta = \frac{T - T_{\text{surr}}}{T_{\max} - T_{\text{surr}}}$ during the cooling process, where T is the temperature of solution at time t during the cooling process from the time point of turning off the 760 nm laser. A gradient τ_s can be calculated from a fitting curve by the equation: $t = -\tau_s \ln \theta$ (Figure 4.2). The value of hS can be obtained from the equation $hS = \frac{mC_p}{\tau_s}$, where m is the mass of solvent and C_p is the heat capacity of solvent.

The energy input Q_{dis} by the solvent and container is defined as $Q_{\text{dis}} = mC_p \Delta T / t$, where m and C_p is the mass and heat capacity of the solvent, ΔT is the increased temperature of the solvent in the container and t is the exposure time by 760 nm laser.

The photothermal conversion efficiency of ATTO740 at 760 nm was calculated to be 19.7% (Table 4.1).

M (g)	0.22	I_0 (W)	1.23
C_p (J K ⁻¹ g ⁻¹)	1.98	A_{760}	0.8
τ_s (s)	103.5	ΔT (K)	0
$T_{\max}$ (K)	341.8	Q_{dis} (W)	0
T_{surr} (K)	293.2	η (%)	19.7

Table 4.1 Values of every parameters in the calculation process of light-to-heat conversion efficiency.

Figure 4.2 Plot of $-\ln\theta$ vs. cooling time t for ATTO740 in DMSO.

4.3.2 Determining the optimal concentration of ATTO740 for PTT

To determine the optimal concentration of ATTO740 in DMSO, three ATTO740 solutions with different concentrations (0.01, 0.1 and 1 mg/mL) in DMSO were

irradiated by 760 nm light and the time-resolved photothermal responses of these three solutions were obtained under ambient temperature. Figure 4.3 shows that the increased temperature of ATTO740 solutions with concentrations of 1, 0.1 and 0.01 mg/mL were 38.9, 36.1 and 10.6 °C, respectively. We chose the concentration of 0.1 mg/mL for ATTO740 solution when antibacterial photothermal activities were conducted.

Figure 4.3 Temperature change profiles of three ATTO740 solutions in DMSO with 1, 0.1 and 0.01 mg/mL.

4.3.3 The conjugation process of ATTO740 onto bacteria

The bioorthogonal conjugation of ATTO740 to *B. subtilis*, VanA and VanB was carried out by first labelling the bacteria with an azide group. This is achieved by incorporating the D-amino acid derivative azido-D-Alanine (ADA) into the peptidoglycan (PG) cell wall. Equipped with the knowledge that ADA-treated bacteria can be easily

functionalized with suitable molecules, two conjugate systems are prepared to enable on-demand covalent bonding of ATTO740 to ADA-treated bacteria using NIR light activation. In this case, we prepared a conjugate DBCO-norbornene, and a system consisting of an ATTO740 dye molecule covalently linked to a dHTz-NH₂ *via* the NHS-ester group of the ATTO740 dye molecule. A Nd³⁺-sensitized upconversion nanocrystal (UCNC) decorated with toluidine blue O (TB) on its surface undergoes upconverted energy transfer from the UCNC to TB when excited by 808 nm light. The photoexcited TB then catalyzes the conversion of the dihydrotetrazine (dHTz) moiety in an ATTO740-dHTz conjugate system to tetrazine (Tz) which undergoes efficient inverse electron demand Diels-Alder reaction with prior attached norbornene molecules on bacterial cell walls.

Figure 4.4 Chemical synthesis process.

4.3.4 Confocal imaging of bacteria conjugated with ATTO740

To demonstrate that ATTO740-dHTz can be readily conjugated to Nb-functionalized bacteria with 808 nm light irradiation in the presence of TB-UCNC. Figure 4.5 showed distinct fluorescence images of Nb-functionalized bacteria after reacting with ATTO740-dHTz with exposure to 808 nm light for 5 min in the presence of TB-UCNC while no clear fluorescence images are seen in this case of bacteria mixed with ATTO740. These results illustrated efficient covalently linkage of ATTO740 to bacterial cell wall.

Figure 4.5 Confocal fluorescence microscopy images of ATTO740 conjugated (A)/mixed (B) *B. subtilis* (top row), ATTO740 conjugated (C)/mixed (D) VanA (middle row) and ATTO740 conjugated (E)/mixed (F) VanB (bottom row). Scale bar: 20 μm .

4.3.5 Photothermal effect of conjugated ATTO740 onto bacteria

The antimicrobial photothermal activity of ATTO740 (0.1 mg/mL) mixed with *B. subtilis*, VanA and VanB separately was examined by counting colony forming units (CFU) after irradiation with 760 nm light for 15 min. 82%, 80.7% and 67% bacterial death were achieved for *B. subtilis*, VanA and VanB, respectively. When ATTO740 was conjugated to bacterial cell wall through the biorthogonal conjugation between Nb-functionalized bacteria and ATTO740-dHTz (the concentration of ATTO740 is 0.1 mg/mL) with exposure to 808 nm light in the presence of TB-UCNC. 96.6%, 96.4%

and 95.7% bacterial death were achieved for *B. subtilis*, VanA and VanB, respectively (Figure 4.6). The CFU results showed that conjugated ATTO740 had a stronger antibacterial photothermal efficacy than mixed ATTO740 at the same concentration.

Figure 4.6 Bacterial colonies of *B. subtilis* (first column), VanA (second column) and VanB (third column) on agar plates for untreated bacteria (top row), bacteria mixed with ATTO740 after 760 nm light irradiation (middle row), and bacteria exposed to TB-UCNC and 808 nm light irradiation (5 min), and irradiated by 760 nm light for 15 min (bottom row). The CFU values are presented above each plate.

4.4 Conclusion

To summarize, an on-demand and efficient strategy of bioconjugating a photothermal agent ATTO740 to drug-resistant bacterial cell wall was presented. NIR light was utilized to induce energy transfer from UCNC to TB to catalyze the conversion of ATTO740-dHTz to ATTO740-Tz. ATTO740 was conjugated to bacterial cell wall through click chemistry reaction between norbornene and tetrazine. The enhanced binding of photothermal agent to bacterial cell wall increased the antibacterial photothermal activity toward bacteria. The antimicrobial photothermal efficiency was raised around 20% with respect to the mixed photothermal agent with bacteria.

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