Winter 2013

Structural and Functional Study of Multidrug Membrane Transporters

Feng Ding
Old Dominion University

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STRUCTURAL AND FUNCTIONAL STUDY OF MULTIDRUG MEMBRANE TRANSPORTERS

by

Feng Ding
B.S. June 2001, Soochow University, P. R. China

A Dissertation Submitted to the Faculty of Old Dominion University in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

OLD DOMINION UNIVERSITY
December 2013

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ABSTRACT

STRUCTURAL AND FUNCTIONAL STUDY OF MULTIDRUG MEMBRANE TRANSPORTERS

Feng Ding
Old Dominion University, 2013
Director: Dr. Xiao-Hong Nancy Xu

Multidrug membrane transporters (efflux pumps) in both prokaryotes and eukaryotes are responsible for ineffective treatment of a wide variety of diseases, including infections and cancer, underscoring the importance of better understanding of their structures and functions for design of effective therapies. Despite extensive studies over decades, their underlying molecular mechanisms remain largely unknown. In this dissertation, we focus on the study of structures and functions of multidrug membrane transporters, including ATP-binding cassette transporter (BmrA) and Resistance-Nodulation-Cell Division transporter (MexA,B-OprM) in gram-positive and gram-negative bacteria (Bacillus subtilis and Pseudomonas aeruginosa), respectively. We fused EGFP with N-terminus and C-terminus of BmrA to construct BmrA-EGFP fusion proteins and characterized their efflux functions in four strains of bacterial cells (Ct-BmrA-EGFP, Nt-BmrA-EGFP, BmrA and ΔBmrA, B. subtilis) using fluorescence microscopy and spectroscopy. The results show that BmrA-EGFP fusion proteins exhibit the similar efflux kinetics of BmrA and retain the efflux functions of BmrA. Modeled structures of the fusion proteins show a highly flexible linker region connecting EGFP with BmrA, suggesting a minimal obstruction of EGFP to BmrA. These two new strains of BmrA-EGFP offer the possibility for one to
visualize and study efflux function and mechanisms of BmrA transporters. We further studied the size-dependent efflux functions of BmrA-EGFP and BmrA using single plasmonic nanoparticle imaging probes and spectroscopy and found size-dependent efflux kinetics of BmrA. Using similar approaches, we fused EGFP with MexA,B-OprM transporters and characterized their efflux functions using fluorescence spectroscopy and single NP plasmonic spectroscopy. The results show that EGFP fused MexA,B-OprM only partially retains its original efflux functions. Structural analysis of fusion protein suggests possible steric hindrance of EGFP toward the conformation changes of MexA,B-OprM, which led to the steric effect upon their efflux functions. These important findings offer new insights into the structural and functional studies of multidrug membrane transporters (efflux pumps), and demonstrate the powerful new nanobiotechnology for better understanding of multidrug resistance.
This dissertation is dedicated to my deceased mother, Mrs. Zhu Xiuying for her unselfish devotion, support over the first twenty five years of my life and to my father, Mr. Ding Jiantai for his support and financial assistance, which made all of this possible.
ACKNOWLEDGMENTS

Most importantly, I would like to thank my mentor, Dr. X. Nancy Xu, for her untiring assistance and guidance throughout my Ph. D. study at Old Dominion University. Without her dedication, this dissertation wouldn't be possible. Her attitude towards scientific research has always inspired me and she set a perfect example for me to follow in my future career.

I would also like to extend my gratitude to the members of my dissertation committee, Drs. Christopher J. Osgood, Lesley H. Greene and Bala Ramjee, for their helpful knowledge, patience and guidance through my graduate education at ODU. In addition, I am grateful to my lab group members for their collaboration and contribution to this dissertation. We have worked together in a wide range of research projects. For example, Prakash Nallathamby synthesized and characterized silver nanoparticles (Ag NPs) used in chapter II, and Kerry Lee led the study of transporters using single NPs in chapter II; Tao Huang synthesized and characterized functional Ag NPs used in chapter V; Ardi Vahedi-Faridi conducted structural analysis of BmrA-EGFP and MexA-EGFP-MexB-OprM fusion proteins. Preeyaporn Songkiatisak and Pavan K. Cherukuri worked with me on the study of effects of functional Ag NPs in chapter V.

This work is supported in part by the National Science Foundation (NIRT: CBET 0507036), National Institute of Health (R01 GM076440), and Old Dominion University.
NOMENCLATURE

Å  Angstrom
ε  Extinction coefficient
µg/mL  Microgram per milliliter
µL  Microliter
µm  Micrometer
µM  Micromolar
Δ  Deletion
Z  Zeta potential in mV
λ  Wavelength
ABC  ATP binding cassette
Ag  Silver
AgClO₄  Silver perchlorate
AgMUNH₂  Silver nanoparticles functionalized with MUNH₂ groups
AgMUNH₂-OFLX  AgMUNH₂ nanoparticles conjugated with ofloxacin
AgNO₃  Silver nitrate
Au  Gold
B. subtilis  Bacillus subtilis
cm  Centimeter
C  Concentration
CCD  Charge coupled device used for imaging nanoparticles with high sensitivity, temporal resolution and spatial resolution
DFOM  Dark field optical microscopy
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<th>Abbreviation</th>
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<tr>
<td>DFOMS</td>
<td>Dark field optical microscopy and spectroscopy</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECFP</td>
<td>Enhanced cyan fluorescence protein</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescence protein</td>
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<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
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<tr>
<td>FWHM</td>
<td>Full width at half maxima</td>
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<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
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<td>H</td>
<td>Hour</td>
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<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HRTEM</td>
<td>High resolution transmission electron microscopy</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani Broth</td>
</tr>
<tr>
<td>LSPR</td>
<td>Localized surface plasmon resonance</td>
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<tr>
<td>MAB</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MCH</td>
<td>6-mercapto-1-hexanol</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
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<tr>
<td>MF</td>
<td>Major facilitator</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<tr>
<td>mm</td>
<td>Millimeter</td>
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<tr>
<td>MSI</td>
<td>Multi-spectral imaging</td>
</tr>
<tr>
<td>MUA</td>
<td>11-Mercatoundecanoic acid</td>
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MUNH$_2$ 11-Amino-1-undecanethiol hydrochloride (AUT)
mV Millivolt
nM Nanomolar
nm Nanometer
NaBH$_4$ Sodium borohydride
NaCl Sodium chloride
NBD Nucleotide binding domain
NFs Nanoframes
NPs Nanoparticles
OD Optical density
OFLX Ofloxacin
P. aeruginosa Pseudomonas aeruginosa
PBS Phosphate buffered saline
pM Picomolar
PVP Polyvinylpyrrolidone
QDs Quantum dots
QY Quantum yield
RFP Red fluorescence protein
RND Resistance nodulation cell division
RPM Revolutions per minute
S Second
SMD Single molecule detection
SMNOBS Single molecule nanoparticle optical biosensors
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<td>SMR</td>
<td>Small multidrug resistance</td>
</tr>
<tr>
<td>S-NHS</td>
<td>N-hydroxysulfosuccinimide</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>T</td>
<td>Temperature in Kelvin</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>UV-vis</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>WT</td>
<td>Wide type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescence protein</td>
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CHAPTER I
OVERVIEW

The phenomena of multidrug resistance (MDR) have been identified in many types of species ranging from bacterium to human, due to existence of different types of efflux pumps such as the major facilitator (MF) superfamily, the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) family and the ATP binding cassette (ABC) superfamily [1-8]. Overexpression of some ABC pumps often causes the failure of clinical chemotherapies in many types of human diseases such as cystic fibrosis (CF) and other cancers [9-11]. Other efflux pumps in the RND family such as MexA,B-OprM and MexC,D-OprJ are also found to be responsible for multidrug resistance in the gram-negative bacteria, *Pseudomonas aeruginosa* [12-17].

Fluorescent proteins [e.g., cyan fluorescent protein (CFP), green fluorescent protein (GFP), yellow fluorescent protein (YFP) and red fluorescent protein (RFP)] have been widely used to study those MDR transporters [18-20]. For example, green fluorescent protein (GFP) has been extensively used as reporter genes and fluorescence probes to study location, distribution and functions of proteins in live cells [21-23]. Fluorescent fusion proteins have been used to label the ABC transporter genes in *Caenorhabditis elegans* and to identify the locations of ABC transporters in fission yeast [24].

Compared to conventional fluorescent proteins as described above, nanometer sized particles such as quantum dots, noble metal nanoparticles
(NPs) (e.g., gold or silver NPs) or magnetic NPs have found their applications in the area of cancer nanotechnology due to their unique optical, magnetic and structural properties which are lacking as compared to biological molecules and bulk materials [25-31]. These properties give them unprecedented advantages for the diagnosis and treatment of cancer and other human diseases when they are conjugated with a variety of diagnostic (e.g., optical or magnetic) and therapeutic (e.g., anticancer drugs or other antibiotics) agents [26, 29, 32-36]. Recent advances are made available in applying NP based probes for molecular and cellular imaging [37-39], targeted cancer therapy [29, 40] as well as early screening and detection of cancer [25, 28, 31, 32, 41]. Among those nanometer sized particles, noble (Ag or Au) NPs have some unique features, such as size- and shape-dependent optical properties, a high surface area to volume ratio [37-39, 42-44]. These optical properties are mainly due to a phenomenon called localized surface plasmon resonance (LSPR), which is highly dependent upon their size, shape and surrounding environments. Besides, noble NPs exhibit strong Rayleigh scattering, allowing them to be directly observed and studied using dark-field optical microscopy and spectroscopy (DFOMS) systems [37-39, 42-44]. All of these properties have made it possible for them to be very promising probes for exploring the unknown mechanisms of MDR transporters.

Though extensive studies have been done on the multi-drug resistance (MDR) transporters over the last two decades, there is still much controversy regarding the exact efflux mechanisms of many well-studied transporters such as PgP and MsbA [3, 5, 8, 45-48]. Thus, it is still crucial to study their efflux
mechanisms by applying new research tools. The potential outcomes from these studies will serve as guidance for the development of effective therapeutic agents for combating those MDR caused diseases.

In this dissertation, I first created two new transgenic strains of *Bacillus subtilis* (*B. subtilis*) by fusing EGFP with a ABC transporter called BmrA in gram-positive *B. subtilis* and expressed them in ΔBmrA strain [22]. Then, we characterized the efflux functions of those fusion strains by using both traditional fluorescence probes and novel nanoparticle optical probes. Next, we created and well characterized the efflux functions of a similar fusion strain by fusing EGFP to MexB antiporter gene and expressing the fusion gene in ΔMexB strain of *Pseudomonas aeruginosa* (*P. aeruginosa*), which is gram-negative bacteria and causes many MDR problems due to the existence of a major RND pump named MexA,B-OprM. After that, we took a step further by using antibiotics conjugated nanoparticles as both optical probes and drug carriers to study the toxicity and biocompatibility of nanoparticle based drug carriers. The obtained outcomes from those studies enriched our knowledge about the structures and functions of MDR transporters as well as offered new insights into the future designing of smart drug carriers to overcome the MDR related problems in terms of increasing targeting efficacy of therapeutic agents.

This dissertation is comprised of six chapters. In chapter I, we provide a general overview of the research background related to our studies as well as the outlines of contents of each chapter.
In Chapter II, in order to study the efflux mechanisms of those MDR transporters, we first need to light up them. Hence, this Chapter describes the molecular cloning and characterization of an EGFP fused ABC transporter (BmrA) in gram-positive Bacillus subtilis [22], as illustrated in Fig. 1. We created two fusion strains by fusing EGFP with both C-terminus and N-terminus of BmrA gene (Fig. 1-A and B) and successfully expressed the fusion genes in ΔBmrA strain. We then characterized the efflux functions of those fusion strains and compared the results with the ones from using WT and ΔBmrA strains and determined that the two new fusion strains maintained the original efflux functions of BmrA transporter while the strain containing N-terminal fusion of EGFP with BmrA functions a little better than the other fusion strain.

In Chapter III, from a different perspective, we probed the efflux functions of the BmrA transporter in different B. subtilis strains including those EGFP fused BmrA strains by using 12 nm Ag NPs as single nanoparticle optical probes [37], as illustrated in Fig. 2. Our results indicated that 12nm Ag NPs are the substrates of this BmrA efflux pump. Besides, different strains showed different efflux kinetics of 12 nm Ag NPs. More specifically, we found ΔBmrA strain accumulated more intracellular NPs over time than WT or the two fusion strain did due to the lacking of BmrA transporter. Those results clearly suggested that the Ag NPs could also be the substrates of those MDR transporters, which makes it possible for us to use them to probe the efflux function kinetics over time owing to their unique physical and chemical properties.
In Chapters IV, to compare with the EGFP fused BmrA transporter in gram-positive *Bacillus subtilis* (Chapter II), we fused EGFP to the MexB part of MexA,B-OprM transporter in gram-negative *Pseudomonas aeruginosa* and well characterized the efflux functions of this new fusion strain, as illustrated in Fig. 3. Our results indicated that EGFP fused MexB antiporter only retained partial efflux functions of MexA,B-OprM efflux pump, which suggest possible steric hindrance effects resulting from either the fusion of EGFP with MexB or the assembly of a dysfunctional MexA,B-OprM efflux pump which contains three copies of each component of this efflux pump (MexA, MexB and OprM) on the inner and outer membranes of *P. aeruginosa* bacteria.

In Chapter V, in order to study the biocompatibility and toxicity of NPs as effective drug carriers, we conjugated silver NPs with the antibiotic (ofloxacin) and compared the size-dependent antibacterial effects of ofloxacin alone (absence of NPs) and various sized silver NPs conjugated ofloxacin in *Pseudomonas aeruginosa*, as illustrated in Fig. 4. Our results indicated that larger sized nano-carriers generated more potent antibacterial effects compared to smaller sized nano-carriers when the total amount of antibiotic molecules contained in all sized nano-carriers is the same. Those interesting results offered new insights into the future design of efficient drug carriers in the process of combating the MDR caused diseases.

In Chapter VI, we summarized our major research findings in those preceding chapters (Chapter II-V).
Fig. 1 Illustration of design and characterization of efflux functions of ABC-EGFP transporters in gram-positive *Bacillus subtilis*.

We fused *EGFP* to both C-terminus and N-terminus of *BmrA* gene and expressed fusion genes in Δ*BmrA* strain. Efflux functions of fusion strains were characterized by time course incorporation of fluorescence dye molecules and results suggested that both fusion strains maintained the original efflux functions of *BmrA* transporter. Modeling prediction of both fusion proteins indicated no steric hindrances effects of *EGFP* to *BmrA* protein. The individual *BmrA* monomers (light orange or yellow), AMP-PNP (red-white-blue spheres), and *EGFP* (green) are illustrated, respectively [22].
Fig. 2 Illustration of transport of silver NPs across the cell membrane by a ABC transporter (BmrA) in gram-positive Bacillus subtilis.

We probed the efflux functions of different B. subtilis strains with different expression level of BmrA proteins by transport of Ag NPs based single plasmonic optical probes across the cell membrane. Results indicated that accumulation and efflux kinetics of intracellular NPs for single living cells depended upon the cellular expression level of BmrA, NP concentrations, and a pump inhibitor (orthovanadate), suggesting that NPs are substrates of BmrA transporters and the passive diffusion driven by concentration gradients is the primary mechanism for NPs to enter the cells. The functional BmrA transporter is illustrated as combination of two BmrA monomers with each monomer containing a nucleotide binding domain (A) and a transmembrane domain (T). Ag NPs are illustrated as blue, green, yellow and red spheres [37]
Fig. 3 Illustration of design and characterization of efflux functions of RND-EGFP transporters in gram-negative *Pseudomonas aeruginosa.*

We fused *EGFP* to N-terminus of *mexB* gene and expressed the fusion gene in Δ*MexB* strain. Efflux functions of fusion strains were characterized by both time course incorporation of fluorescence dye molecules and measurement of MICs of selected antibiotics. Results suggested that the fusion strain partially retained the original efflux functions of MexA,B-OprM transporter. Modeling prediction of the fusion protein indicated possible steric hindrances effects of *EGFP* fused to MexB, which may lead to dysfunctional assembly of MexA,B-OprM transporter across the cell membrane. The membrane fusion protein MexA (turquoise), outer membrane channel OprM (violet), the individual antiporter MexB monomers (red, orange, and light brown) and *EGFP* (green) are illustrated, respectively [8]
Fig. 4 Illustration of study of size-dependent antimicrobial effects of nano-carriers on gram-negative bacteria, *Pseudomonas aeruginosa*.

We synthesized and characterized three sized Ag NPs conjugated with drug molecules and we studied the antimicrobial effects of these nano-carriers on the growth of bacteria in *P. aeruginosa*. We found the size-dependent inhibitory effects of these nano-carriers on the growth of bacteria, with larger nano-carriers conferring higher antimicrobial effects than smaller ones at the same concentration of conjugated drug molecules on their surfaces. Ag NPs of different sizes (2.4, 13.0 and 92.6 nm) are illustrated as blue, green and red spheres, respectively. Molecular structure of ofloxacin is shown above and ofloxacin in different sized Ag carriers is represented by 4-point star (٭)
INTRODUCTION

ABC (ATP-binding cassette) transporters exist in all live organisms, ranging from bacteria to human [1, 49-53]. They are involved in the unidirectional transport, either import or export, of a wide variety of structurally and functionally unrelated substrates, including ions, sugars, lipids, amino-acids, proteins, or xenobiotics, against substrate concentration gradients [1, 49-54]. Such function is responsible for multidrug resistance (MDR) in bacteria and cancer cells. In bacteria, ABC transporters constitute the largest protein family. For instance, 78 ABC transporters have been reported in *B. subtilis* [55, 56]. Notably, 48 ABC transporters have been identified in human. Abnormal functioning of some of these ABC transporters in human is directly responsible for severe pathologies [10]. For example, P-glycoprotein, the first mammalian ABC transporter identified, accounts for MDR in cancer cells, which leads to ineffective therapy. Cystic fibrosis is caused by mutations in the chloride channel of cystic fibrosis transmembrane regulator [9-11].

Interestingly, all ABC transporters share a common organization, which consists of four core domains: two transmembrane domains (TMD) and two nucleotide-binding domains (NBD) [46, 52, 57-61]. The TMDs are quite variable
in sequence and topology, which defines the substrate binding sites and forms the transport passageway for substrates to cross the membrane. In contrast, the NBDs show conserved sequences, which empower the transporters by binding and hydrolyzing ATP [46, 57, 61, 62]. These four domains are either fused as a single polypeptide or present in two to four separate polypeptides. Three-dimensional (3D) structures of many NBDs isolated from various origins share the same global topology, suggesting a common evolutionary origin and similar mechanisms of energy coupling in all ABC transporters [52, 62].

Despite extensive studies, their translocation mechanisms remain elusive [9, 52]. Many fundamental questions about molecular mechanisms and interactions of multidrug ABC transporters (efflux pumps) remain unanswered [9, 52, 62]. For example, one major question is how to identify the individual integrated steps of the catalytic cycle, which are responsible for substrate translocation. Notably, fusion of ABC membrane transporters with EGFP offers the opportunity to visualize membrane transporters and to study their interactions with substrates that lead to their translocations. However, the fusion of ABC transporters with EGFP may alter their efflux function. Thus, it is crucial to first characterize the efflux function of ABC-EGFP transporters, prior to using them to probe their molecular mechanisms.

Fluorescent proteins (e.g., GFP) have been widely used as reporter genes and non-invasive fluorescence probes for study of molecular mechanisms and functions of proteins in live cells [20, 63]. Promoter-fluorescent protein fusions have been used to identify the ABC transporter genes in Caenorhabditis elegans
and to determine the locations of ABC transporters in fission yeast [24, 64]. GFP has been used as a fluorescence probe to characterize the topologies of ABC transporters, because GFP fluoresces in the cytoplasmic space (reducing environment), but not in the periplasm or plasma membrane [65, 66]. Studies have shown that GFP fusion proteins (membrane transporters) may alter the function of proteins [67, 66]. Unfortunately, the functions of GFP fused proteins (transporters) have not yet been widely characterized and compared with the non-fused proteins.

Currently, structures of membrane transporters are characterized by X-ray crystallography, which is limited by the difficulties of crystallization of membrane proteins. It is also unable to provide real-time kinetic information about the molecular mechanisms and conformation change of ABC transporters.

The primary methods for study of multidrug membrane transporters in bacterial and mammalian cells include the measurement of their accumulation of radioactively labeled substrates (\(^{14}\)C and \(^{3}\)H) and the fluorescence dyes (e.g., rhodamine 123, Fluo-3, Hoechst dyes) [54, 68-76]. Fluorescent dyes, such as Hoechst dyes and ethidium bromide (EtBr), emit weak fluorescence in aqueous solution (outside cells) and become strongly fluorescent in non-polar and hydrophobic environments, especially as the dye molecules enter cells and intercalate with DNA [77]. Thus, time-dependent (time-course) fluorescence intensity of the dyes is particularly suitable for real-time monitoring of the accumulation of substrates in bacterial and mammalian cells and for study of multidrug transporters in live cells [54, 68-76].
Efflux function of multidrug membrane transporters has also been characterized by determining the minimal inhibitory concentrations (MICs) of drugs (e.g., antibiotics) that are specific substrates of the transporters and show the specific susceptibility toward given strains of bacterial cells [78, 79]. Unfortunately, to our knowledge, antibiotics that are specific substrates of BmrA membrane transporter and show specific susceptibility for BmrA cells, but not ΔBmrA cells, have not yet been identified. Thus, MIC method has not yet been used to characterize BmrA transporter.

In this study, we constructed two fusion proteins by fusing either C- or N-terminal BmrA gene with EGFP vectors, and expressing them in ΔBmrA cells to produce two new strains of B. subtilis. To determine whether BmrA-EGFP transporters retain the efflux function of BmrA, we characterized their efflux kinetics of fluorescence molecules (Hoechst 33342), and their dependence on the expression of BmrA and the presence of a pump inhibitor (orthovanadate) using fluorescence spectroscopy. To our knowledge, BmrA-EGFP transporters in B. subtilis have not yet been reported. These new strains are needed for study of functions and structures of BmrA transporter in single live cells.

RESULTS AND DISCUSSION

Construction and characterization of BmrA-EGFP fusion genes and their expression

We designed and constructed bmrA-EGFP fusion genes by fusing the C-terminal or N-terminal bmrA genes with EGFP vectors. The fusion genes,
pSG1154-BmrA (54A) and pSG1729-BmrA (29A), were constructed as shown in Fig. 5 and as described in Experimental Section. The pET23-BmrA plasmid was amplified using two sets of primers, C-terminal (CP1, CP2) or N-terminal (NP1, NP2) primers, via PCR. The amplified PCR products and vectors (pSG1154 and pSG1729) were then digested using a pair of restriction endonuclease enzymes (XhoI/EcoRI). The digested and purified PCR products (1800 base pairs, bp, bmrA genes) were finally ligated with the digested and purified vectors (7600 bp), pSG1154 or pSG1729, respectively. This approach enables us to insert the bmrA gene into each vector via fusion of C-terminal or N-terminal bmrA genes with EGFP gene, and to create two fusion genes (pSG1154-BmrA and pSG1729-BmrA), respectively.

The amplified PCR products in Fig. 5 were characterized using gel electrophoresis and DNA sequencing. The results in Fig. 6A show that both PCR products of pET23-BmrA plasmid amplified by using C-terminal and N-terminal primers are 1,800 bp, which agrees well with the number of base pair of the bmrA gene [80]. The fusion genes digested by a pair of restriction endonuclease enzymes (XhoI/EcoRI) (Fig. 6B) show the products of 7,600 bp and 1,800 bp, which agree well with that of digested vectors and bmrA genes [18, 80], respectively. The DNA sequencing results (sequence data not shown) of both fusion genes show that the sequences of bmrA gene are in excellent agreement with those reported previously [18, 80].
Fig. 5 Schematic illustrations of construction of (A) Ct-bmrA-EGFP and (B) Nt-bmrA-EGFP fusion genes.

The plasmid (pET23-BmrA) was amplified using two pairs of primers (CP1 and CP2, or NP1 and NP2) by PCR, respectively. The amplified genes and vectors (pSG1154 or pSG1729) were digested by restriction enzymes (Xhol/ EcoRl). The digested genes were ligated to the digested vectors, generating pSG1154-BmrA (54A, Ct-bmrA-EGFP) and pSG1729-BmrA (29A, Nt-bmrA-EGFP) fusion genes, respectively.
Fig. 6 Characterization of \textit{bmrA-EGFP} fusion genes using agarose gel electrophoresis.

(A) L1: DNA markers/ladders in base pair (bp); L2: \textit{bmrA} gene amplified using the plasmid (pET23-BmrA) as a template and CP1 or CP2 as primers (PCR Product I in Fig. 5); L3: \textit{bmrA} gene amplified using the plasmid (pET23-BmrA) as a template and NP1 or NP2 as primers (PCR Product II in Fig. 5). Arrows point to the PCR products of 1,800 bp, which agrees with the number of base pairs of \textit{bmrA} genes. (B) L1: DNA markers in bp; L2: pSG1729-BmrA; L3: pSG1154-BmrA; L4: pSG1729, and L5: pSG1154, digested by Xhol/EcoR, respectively. Arrows point to the digested vector plasmids at 7,600 bp and \textit{bmrA} genes at 1,800 bp
Fig. 7 Characterization of expression of bmrA-EGFP fusion genes in ΔBmrA cells.

(A) Dark-field optical images and (B) green fluorescence images of single live cells: (a) Nt-BmrA-EGFP; (b) Ct-BmrAEGFP; (c) WT-BmrA; and (d) ΔBmrA strains, respectively. The intensity scales are 55–355 ADC (analog and digital counts) in (A) and 55–95 ADC in (B) Scale bar = 5 μm

We transformed the fusion gene (pSG1154-BmrA or pSG1729-BmrA) into ΔBmrA cells to create two new strains of B. subtilis (Ct-BmrA-EGFP and Nt-BmrA-EGFP cells). Their expression in single live cells was characterized by measuring fluorescence of EGFP in single live cells using fluorescence microscopy and spectroscopy. The results in Fig. 7 show that all Ct-BmrA-EGFP
and Nt-BmrA-EGFP cells, but none of BmrA and ΔBmrA cells, emit green fluorescence, which demonstrates the successful expression of fusion genes in the cells.

Notably, these two new strains offer the possibility of characterization of locations, topologies and functions of BmrA membrane transporters in live cells, as those reported for other ABC transporters [65-67, 81]. For instance, fusion of EGFP with BmrA enables one to probe the locations and topologies of BmrA and the translocation of pump substrates (efflux function) of BmrA in live cells in real time. However, BmrA-EGFP transporters must first be characterized to determine whether they retain the original efflux functions of BmrA, prior to their uses for study of functions and structures of BmrA.

Study of suitability of fluorescence molecules for probing of efflux kinetics of transporters in live cells

Hoechst dyes are well known substrates of ABC membrane transporters and have been used to monitor the accumulation and efflux kinetics of the BmrA transporter embedded in vesicles [54, 76]. Notably, Hoechst molecules emit weak fluorescence in aqueous solution (outside the cells), and their fluorescence intensity increases substantially (up to 10-fold), as they enter into the cells and intercalate with DNA [77]. Thus, the fluorescence intensity of the dye molecules can be used to measure the accumulation and efflux kinetics of membrane transporters in live cells in real time.
To determine whether time-dependent fluorescence intensity of the dye molecules acquired at the peak wavelength ($\lambda_{\text{max}}$) of 450 nm is suitable for monitoring their accumulation kinetics in live cells in real time, we acquired the fluorescence spectra of the dye molecules incubated with the live cells (Ct-BmrA-EGFP, Nt-BmrA-EGFP, WT, ΔBmrA cells) over time. The representative results in Fig. 8A show that the $\lambda_{\text{max}}$ of its emission spectra at 450 nm remains essentially unchanged over time, and its fluorescence intensity increases with time. The results show that intercalation of dye molecules with intracellular DNA in live cells did not lead to the shift of $\lambda_{\text{max}}$ of their excitation and emission spectra, and the presence of EGFP did not affect fluorescence measurements of the dye molecules.

To determine whether photodecomposition of the dye molecules could significantly affect their fluorescence intensity over time, we measured the excitation and emission spectra of the dye molecules incubated with the live cells that had been constantly irradiated by the excitation beam (350 nm) or kept in the dark for 30 min. The results in Fig. 8B show that the fluorescence intensity and $\lambda_{\text{max}}$ of the excitation and emission spectra for the exposed and unexposed samples are nearly identical.

Taken together, the results in Fig. 8 show that the time-dependent fluorescence intensity of the dye molecules incubated with live cells at 450 nm is well suited for monitoring of their accumulation kinetics in live cells using fluorescence spectroscopy. By probing of dependence of their accumulation kinetics on the expression level of BmrA in each strain, we can characterize the
efflux function of BmrA and BmrA-EGFP transporters in live cells and determine whether BmrA-EGFP retains the efflux function of BmrA.

**Probing of dependence of efflux kinetics of transporters on their expression in live cells**

To determine whether BmrA-EGFP proteins expressed in ΔBmrA cells retain the efflux function of BmrA transporter, we studied the efflux kinetics of membrane transporters in live cells in real time using the fluorescence probe (Hoechst 33342). The studies were performed in the absence and presence of glucose, aiming to determine the roles of glucose on the efflux function of BmrA in live cells. It is worthy noting that previous studies for probing of accumulation and efflux kinetics of the dye molecules for ABC transporters were carried out in the presence of glucose [54, 76]. Notably, the potential effects of the glucose on the accumulation and efflux function of ABC transporters were not studied.

The results in Fig. 9A show that, in the absence of glucose, the fluorescence intensity of the dye molecules increases with time, indicating that the dye molecules enter the cells and intercalate with intracellular DNA. Notably, the accumulation rates (slope of the each curve) highly depend upon the expression level of BmrA in the cells. ΔBmrA cells (deletion of BmrA) show the highest accumulation rates with the highest fluorescence intensity at each given equilibrium time, while WT-BmrA cells (normal expression of BmrA) exhibit the lowest rates.
Fig. 8 Characterization of fluorescence properties of Hoechst dye molecules for probing the efflux kinetics of BmrA and BmrA-EGFP membrane transporters in live cells (Ct-BmrA-EGFP or Nt-BmrA-EGFP).

(A) Fluorescence emission spectra of the dye molecules (2 μM) incubated with the cells (OD_{600 nm} = 0.1) at (a) 5, (b) 200, and (c) 400 s, show that the peak wavelength of the spectra (λ_{max} = 450 nm) remains essentially unchanged, while fluorescence intensity increases with time. (B) Excitation and emission spectra of the dye molecules (2 μM) incubated with the cells that: (a) and (b) had been exposed to 350 nm excitation beam for 30 min; and (c) and (d) in the dark, show that the fluorescence intensity and λ_{max} of the spectra of both samples are nearly identical.
Both Nt-BmrA-EGFP and Ct-BmrA-EGFP cells (over expression of BmrA) show the lower accumulation rates than ΔBmrA, suggesting that both BmrA-EGFP cells extrude the intracellular dye molecules which lead to the lower accumulation rates. Notably, Nt-BmrA-EGFP cells have slightly higher accumulation rates than BmrA cells, but much lower accumulation rates than Ct-BmrA-EGFP cells. The results suggest that fusion of either N-terminal or C-terminal BmrA with EGFP retains efflux function of BmrA, with less steric effect of EGFP on N-terminal fusion.

In the presence of glucose (Fig. 9B), ΔBmrA cells display the highest accumulation rates with the highest intracellular dye molecules, both Ct-BmrA-EGFP and Nt-BmrA-EGFP cells show the lowest rates with the lowest amount of the intracellular dye molecule. Interestingly, the BmrA cells show higher accumulation rates with higher amount of intracellular dye molecules than BmrA-EGFP cells. The results show that the presence of glucose leads to three orders of magnitude higher accumulation rates and higher amount of intracellular dye molecules in a shorter period of time than the absence of glucose. The quantitative results of Fig. 9 are summarized in Table 1.

Taken together, the results in Fig. 9 indicate that the presence of glucose affects the study of the efflux function of BmrA and BmrA-EGFP transporters using the dye molecules via fluorescence spectroscopy. The results suggest drastic effects of glucose upon the accumulation and efflux of the dye molecules by live cells. Further studies are underway to determine the roles of glucose in accumulation and efflux function of BmrA transporters.
Fig. 9 Study of the dependence of accumulation and efflux kinetics of the intracellular dye molecules on the expression of BmrA and BmrA-EGFP transporters in live cells.

Time-dependent fluorescence intensity of the dye molecules (2 μM) incubated with cells (OD\textsubscript{600 nm} = 0.1): (a) ΔBmrA, (b) Ct-BmrA-EGFP, (c) WT (BmrA), and (d) Nt-BmrA-EGFP strains, in (A) absence and (B) presence of 25 mM glucose.
Inhibitory effects of a pump inhibitor on efflux kinetics of transporters in live cells

To determine whether BmrA and BmrA-EGFP membrane transporters are specifically responsible for the efflux of the dye molecules out of the cells, we studied the accumulation kinetics of the intracellular dye molecules in live cells (WT BmrA, Nt-BmrA-EGFP, Ct-BmrA-EGFP, ΔBmrA cells) in the presence of a pump (ATPase) inhibitor, orthovanadate [82, 83], using fluorescence spectroscopy.

The results in Fig. 10a show, in the absence of the glucose, the lower accumulation rates and the lower amount of the intracellular dye molecules were found in the cells (WT, Nt-BmrA-EGFP, Ct-BmrA-EGFP strain) in the absence of orthovanadate (Fig. 10A-C, a, i) than its presence (Fig. 10A-C, a, ii), respectively. In contrast, the accumulation rates and amount of the intracellular dye molecules for ΔBmrA cells remain nearly unchanged in the absence (Fig. 10D, a, i) and the presence of the orthovanadate (Fig. 10D, a, ii). This experiment serves as a control, which demonstrates that orthovanadate at the given dose used in this study specifically inhibits the efflux function of BmrA transporter, but not other membrane transporters in the cells.

These results show the inhibitory effects of the pump inhibitor on the efflux function of BmrA and BmrA-EGFP, suggesting that BmrA and BmrA-EGFP transporters are indeed responsible for the extrusion of the dye molecules out of the cells, which lead to the lower accumulation rates than ΔBmrA. Interestingly, the less inhibitory effects of orthovanadate on efflux of the dye molecules by Nt-
BmrA-EGFP than Ct-BmrA-EGFP (Fig. 10B-C, a) were observed. The plausible explanation may be that either high amount of orthovanadate are needed to inhibit the efflux function of Nt-BmrA-EGFP or the N-terminal fusion is less sensitive to the inhibitory effect of orthovanadate than C-terminal fusion. Further studies are needed to depict the detailed mechanisms.

Interestingly, in the presence of glucose, the accumulation rates of the dye molecules and their accumulation amount in all four strains remain essentially unchanged in the presence and absence of orthovanadate, as shown in Fig. 10b, respectively.

Notably, the accumulation rates of the intracellular dye molecules are much lower in the absence of glucose (Fig. 10a) than its presence (Fig. 10b). The quantitative analysis of accumulation rates in Fig. 10 are summarized in Table 1. The results suggest that the effects of glucose on the accumulation and efflux of the dye molecules in live cells are higher than the inhibitory effects of orthovanadate. Thus, in the presence of glucose, we observed no inhibitory effects of orthovanadate.

Taken together, these results (Fig. 10) show the inhibitory effect of orthovanadate upon efflux function of BmrA and BmrA-EGFP transporters and suggest that they are responsible for efflux of the dye molecules out of the cells. Thus, BmrA-EGFP retains the efflux function of the BmrA. The results also show the drastic effects of glucose upon the efflux of intracellular dye molecules by the transporters in live cells, suggesting that efflux function of ABC transporters must be characterized in the absence of glucose.
Table 1 Summary of accumulation rates of dye molecules in live cells

<table>
<thead>
<tr>
<th>C&lt;sub&gt;glucose&lt;/sub&gt; (mM)</th>
<th>Inhibitor&lt;sup&gt;a&lt;/sup&gt; (μM)</th>
<th>Accumulation Rate&lt;sup&gt;b&lt;/sup&gt; (1/s)</th>
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<tr>
<td></td>
<td></td>
<td>WT</td>
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<tr>
<td>0</td>
<td>0</td>
<td>3.8 x10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>25</td>
<td>2.2 x10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>1.1</td>
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<tr>
<td>25</td>
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</table>

<sup>a</sup> Orthovanadate

<sup>b</sup> Rate = slopes of the curves at its half equilibrium time. The equilibrium time is defined at the time that the fluorescence intensity reaches the maximum and remains essentially unchanged.

<sup>c</sup> Ct-BmrA-EGFP: cells with the over-expression of C-terminal BmrA fused with EGFP

<sup>d</sup> Nt-BmrA-EGFP: cells with the over-expression of N-terminal BmrA fused with EGFP

Structural Modeling and Analysis of Fusion BmrA-EGFP Transporters

The results in Figs. 9A and 10a show that Ct-BmrA-EGFP and Nt-BmrA-EGFP transporters extrude the intracellular dye molecules and their efflux function is inhibited by the ABC (ATPase) inhibitor (orthovanadate), suggesting
Fig. 10 Probing of inhibitory effects of a pump inhibitor (orthovanadate) on efflux function of BmrA and BmrA-EGFP transporters.

Time-dependent fluorescence (FL) intensity of the dye molecules (2 μM) incubated with the cells (OD_{600nm} = 0.1): (A) WT (BmrA), (B) Ct-BmrA-EGFP, (C) Nt-BmrA-EGFP, and (D) ΔBmrA, in (a) absence and (b) presence of glucose (25 mM) and (i) without and (ii) with orthovanadate (25 μM). Note that the initial fluorescence intensity in Fig. 10D-a varies slightly, which is attributed to the slight variation of cell concentrations.
that the fusion proteins retain efflux function of BmrA. The results show that Nt-BmrA-EGFP transporter exhibits the higher efflux rates (lower accumulation rates) than Ct-BmrA-EGFP, indicating that EGFP contributes less hindrance to the efflux function of Nt-BmrA-EGFP than Ct-BmrA-EGFP transporter.

To better understand these interesting findings, we modeled Ct-BmrA-EGFP and Nt-BmrA-EGFP, as described in experimental and model section. We used SWISS-MODEL to assemble EGFP and BmrA models using available 3D structures of GFP (PDB-code: 3ogo) with a sequence identity of 98.7%; and homologous structural models of the 'closed' multidrug ABC transporter Sav1866 and LmrA with up to 33.7 % sequence identity (PDB-codes: 2onj, 2hyd, 1mv5) [84, 85], respectively. The secondary structures of the truncated regions connecting the EGFP to BmrA were analyzed by PSIPRED server [86]. The results (Fig. 11A-B) show that the linker regions are unstructured and conformationally flexible.

The model of Ct-BmrA-EGFP dimer in Fig. 11C shows that the EGFP situates at the top of the ATP-binding domain (NBD) of the BmrA transporter. In contrast, the model of Nt-BmrA-EGFP dimer in Fig. 11D depicts that the EGFP protrudes sideways from the TMDs. We observed fluorescence of EGFP for both fusion strains, indicating that the EGFP was located in the cytoplasm, but not in the cellular plasma membrane (next to the TMD). Studies have shown that, in contrast to cellular plasma membranes, the cytoplasmic space offers the required reducing conditions, which enable the EGFP to fluoresce [65].
Fig. 11 Illustrations of modeled structures of Ct-BmrA-EGFP and Nt-BmrA-EGFP proteins expressed in ΔBmrA cells.

Secondary structures of the fusing regions of (A) N- and (B) C-terminal BmrA with EGFP show unstructured and highly flexible linker regions. (C) Ct-BmrA-
EGFP dimer model shows the EGFP domains on the top of the ATP-binding domains (NBDs). (D) Nt-BmrA-EGFP dimmer model shows the EGFP domains protruding sidewards of transmembrane domains (TMDs). The individual BmrA monomers in light orange or yellow, AMPPNP in red–white–blue spheres, and EGFP in green are illustrated.

Both structures show that the EGFP is linked to BmrA via a long unstructured loop region comprised of 19 amino acids for the Ct-BmrA-EGFP and 23 amino acids for the Nt-BmrA-EGFP, respectively. Such highly flexible linker regions allow substantial mobility of the EGFP molecule in the positional placement in relation to the BmrA transporter. Thus, EGFP does not create significant obstruction to the BmrA in either fusion proteins. Nonetheless, the structure of Ct-BmrA-EGFP dimer (Fig. 11C) shows the closer proximity (~14 Å) of two C-termini of BmrA in the “closed” dimer. Thereby, it may result in additional interference of EGFP with the function of BmrA, as BmrA converts from the ‘open’ to the ‘closed’ conformation during the substrate transport cycle. In contrast, the structure of Nt-BmrA-EGFP dimer (Fig. 11D) illustrates that the distance between two N-termini of BmrA in the “closed” dimer is 47.9 Å, which is larger than that of C-terminal fusion, suggesting less interference of N-terminal fusion of BmrA with EGFP.

Taken together, this structural analysis provides interesting evidence to explain why Ct-BmrA-EGFP and Nt-BmrA-EGFP transporters retain the efflux
function of BmrA, and why Nt-BmrA-EGFP is more favorable for the efflux substrate translocation than Ct-BmrA-EGFP.

SUMMARY

In summary, we have designed and constructed bmrA-EGFP fusion genes and successfully expressed them in ΔBmrA cells to generate two new strains of B. subtilis (Ct-BmrA-EGFP and Nt-BmrA-EGFP). We studied the dependence of accumulation kinetics of intracellular fluorescence molecules (Hoechst 33342, a pump substrate) on the expression level of BmrA and BmrA-EGFP transporters for each strain (WT, ΔBmrA, Ct-BmrA-EGFP, Nt-BmrA-EGFP), by measuring the time-dependent fluorescence intensity of the intracellular dye molecules using fluorescence spectroscopy. We found that either Ct-BmrA-EGFP or Nt-BmrA-EGFP extruded the pump substrate, leading to the lower accumulation of the intracellular dye molecules than ΔBmrA. The results suggest that the fusion proteins retain the efflux function of BmrA. Notably, Nt-BmrA-EGFP extruded the intracellular dye molecules more rapidly than Ct-BmrA-EGFP. Our structural analysis shows the highly flexible linker regions connecting EGFP with BmrA in the fusion proteins, suggesting a minimal obstruction of EGFP to the BmrA. Nonetheless, two C-termini of the "close" dimer of BmrA is in closer proximity (14 Å) than two N-termini of the dimer (47.9 Å), which suggests the less steric effect of the N-terminal BmrA fusion on its efflux function than the C-terminal fusion. The efflux function of both BmrA-EGFP transporters was inhibited by the ABC pump inhibitor (orthovanadate), which further demonstrated that both fusion
pumps retain the efflux function of BmrA transporter. This study also shows that the presence of glucose affects the accumulation and efflux of the dye molecules by the transporters in live cells using fluorescence spectroscopy. Thus, the efflux function of ABC transporters must be characterized in the absence of glucose.

METHODS

Reagents and cell strains

*Escherichia coli* DH5α (Invitrogen), PCR polymerase (Agilent Technologies), plasmid isolation kit (Qiagen), DNA gel extraction kit (Qiagen), DNA ligation kit (T4 DNA ligase and ligation buffer) (Roche), XhoI/EcoRI (NEB), tetracycline (Calbiochem), spectinomycin (Enzo), xylose (Spectrum), orthovanadate (Sigma), Hoechst 33342 (Invitrogen), casamino acid (BD), tryptose blood agar base powder (BD), *B. subtilis* strain (1A772, WT BmrA) and plasmids (pSG1154 and pSG1729) from Bacillus Genome Stock Center (BGSC) [18], were purchased and used as received. ΔBmrA and plasmid (pET23-bmrA) were provided by J. M. Jault [54].

All other media and cell culture plates were prepared in our lab. Medium A (100 mL) was prepared by mixing 10x Medium A base (10 mL) and 10x Bacillus salts (9 mL) in the sterilized DI water. Medium B were prepared by mixing Medium A (10 mL), CaCl₂ (0.1 mL, 50 mM) and MgCl₂ (0.1 mL, 0.25 mM). The 10x Medium A base (1 L) was prepared by mixing yeast extract (10 g), casamino acids (2 g) and glucose (100 mL, 50%) in DI water. The 10x Bacillus salts (1 L) was prepared by dissolving (NH₄)₂SO₄ (20 g), K₂HPO₄·3H₂O (183 g), KH₂PO₄
(60 g), sodium citrate (10 g), and MgSO$_4$·7H$_2$O (2 g) in DI water. The tryptose blood agar base plate was prepared by mixing tryptose blood agar base powder (3.3 g) in DI water (100 mL), which was then autoclaved, cooled to 45-50 °C, and mixed with sterile defibrinated blood (50 mL). The mixture was immediately used to prepare the plates.

**Cell culture and assay**

Four *B. subtilis* strains (WT-BmrA, Ct-BmrA-EGFP, Nt-BmrA-EGFP, and ΔBmrA cells) were pre-cultured by inoculating single clones of each strain from the tryptose blood agar plates into each flask containing LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.2). The flasks were placed in a shaker (LabLine Orbit-Environ, 37°C, 150 rpm) for 12 h. The cells were then cultured in the medium for another 8 h, WT and ΔBmrA in LB medium and Ct-BmrA-EGFP and Nt-BmrA-EGFP cells in LB medium with 1% xylose. The cultured cells were harvested and washed three times with PBS buffer using centrifugation (Beckman J2-21, JA-14, 6000 rpm). The washed cells re-suspended in the buffer (OD$_{600\ nm} = 0.1$) were used to study the accumulation kinetics of the fluorescence dye (Hoechst 33342) in the cells using fluorescence spectroscopy.

**Construction and characterization of bmrA-EGFP fusion genes**

We fused C-terminal *bmrA* gene with N-terminus EGFP (pSG1154) or N-terminal *bmrA* gene with C-terminus EGFP (pSG1729) in each vector to
construct two fusion genes, \textit{Ct-bmrA-EGFP} (pSG1154-BmrA) and \textit{Nt-bmrA-EGFP} (pSG1729-BmrA) genes (Fig. 5), respectively. We named them as 54A and 29A, respectively.

The pET23-BmrA plasmid was used as a template, and two pairs of primers (CP1 and CP2 or NP1 and NP2) were used to amplify the \textit{bmrA} gene using PCR to generate PCR product I and II (Fig. 6). The sequences of the primers are CP1 (5'-GCG CGC TCG AGG GAG GAA AAT ATA TGC CAA CCA AGA AAC AAA-3'); CP2 (5'-GCG CGG AAT TCC CCG GCT TTG TTT TCT AAG-3'); NP1 (5'- GCG CGC TCG AGA TGC CAA CCA AGA AAC AAA-3'); and NP2 (5'-GCG CGG AAT TCT TAC CCG GCT TTG TTT-3').

Each PCR product and vector (pSG1154 or pSG1729) was digested by a pair of restriction enzymes (\textit{XhoI/EcoRI}) by incubating the PCR product (10 ng) or the vector (5 ng), with two units of \textit{XhoI/EcoRI}, in the digestion buffer (40 or 30 µL) at 37 °C for 3 h. The digested products were characterized using agarose gel electrophoresis (0.8%, 8-10 V/cm) and purified using the gel extraction kit. The digested and purified PCR product I or II (20 nM) were ligated into the digested and purified vector (pSG1154 or pSG1729, 5 nM) in T4 DNA ligase (one unit in 20 µL ligation buffer) at 12 °C overnight, respectively.

Each ligated product was transformed into DH5\textalpha competent cells (Invitrogen), as described previously [87, 88]. Briefly, the ligated solution (10 µL) with the competent cell suspension (100 µL, \sim2x10^7 cells/mL) was mixed and placed on ice for 30 min. The mixture was heated at 42 °C for 90 s and then placed on the ice for another 2 min. The LB media (1 mL) was added into the
mixture, which was incubated in the shaker (37 °C, 150 rpm) for 1 h. Transformed cells were then harvested using centrifugation and suspended in the fresh medium, which was then inoculated onto LB plates and incubated at 37 °C for 24 hours.

The clones from each plate were inoculated into the LB medium (3 mL) containing 100 μg/mL ampicillin and cultured in the shaker (150 rpm, 37 °C) for 24 h. The plasmid (fusion genes) of each clone was isolated and amplified by PCR. The amplified PCR products were characterized using the restriction enzymes (Xhol/EcoRI), gel electrophoresis, and DNA sequencing (Applied Biosystem, 3730xl DNA Analyzer), to verify successful construction of fusion genes.

Characterization of expression of fusion genes in single live cells

Each characterized fusion plasmid was transformed into ΔBmrA cells using a previously reported protocol [87, 88]. Briefly, the ΔBmrA cell plates were prepared by inoculating the cells onto the tryptose blood agar base plates and incubating them at 37 °C overnight. The clones on the plates were inoculated into Medium A (4.5 mL). The cell suspension (OD_{650 nm} = 0.1) was incubated in the shaker (37 °C, 150 rpm) for 3 h. The cell growth was followed over time. The number of the cells increased with time slowly for 40 min, then logarithmically for 50 min and slowly again for another 90 min.

By then, the cell suspension (50 μL) was transferred into pre-warmed Medium B (0.45 mL) and cultured in the shaker (37 °C, 150 rpm) for 90 min. The
fusion plasmid (pSG1154-bmrA or pSG1729-bmrA, 1 μg) was then added into the cell suspension and incubated in the shaker (37 °C, 150 rpm) for 30 min. Each aliquot of the transformants (200 μL for each plate) was spread onto the LB agar plate and incubated at 37 °C for 24 h. The clones were picked and cultured, as described above.

The starch-iodine assay was used to confirm the insertions of bmrA-EGFP fusion genes into the chromosome via double crossover events at the amyE site, as described in the following. Transformants were patched onto the LB agar plates containing 1% (w/v) starch, tetracycline (5 μg/mL) and spectinomycin (50 μg/mL). The plates were incubated at 37 °C over night and stained by vaporizing a few iodine crystals in the inverted plate lids. The blue clones stained by the iodine had their amylase deficient phenotypes, which were correct constructs. The insertions of fusion genes into the chromosome amyE locus had led to the disruption of the amyE gene and dysfunctional AmyE protein, which could not disintegrate the starch.

Note that AmyE protein (α-amylase) can break down starch into small structural units (e.g., maltotriose and maltose). Thereby, the presence of AmyE protein would have failed the starch-iodine test (colorless). The clones showed blue in the starch-iodine test, confirming the successful insertion of each BmrA-EGFP fusion gene into the chromosomal amyE locus. Furthermore, these two new strains fully grew in the medium, and reached confluent at the same time as WT-BmrA and ΔBmrA strains. The results suggest non-clonal effects.
The expression of fusion genes in single live cells was characterized by measuring fluorescence of EGFP. The cell suspension in the PBS buffer were imaged in a micro-chamber using dark-field optical and epi-fluorescence microscope (Nikon, E-400), equipped with a CCD camera (Micromax, Roper Scientific). We have fully described the design and construction of the micro-chamber and dark-field optical and epi-fluorescence microscope for imaging of single cells in our previous studies [37, 38, 73, 75, 89, 90]. In this study, the fluorescence filter cube (Chroma Tech) containing a band-pass excitation filter (455 ± 30 nm), band-pass emission filter (525±30 nm) and a dichroic mirror (500 nm), was used for fluorescence imaging of expression of BmrA-EGFP.

**Fluorescence spectroscopic study of accumulation kinetics of dyes in live cells**

Fluorescence intensity of Hoechst 33342 (2 μM) incubated with the cells (OD$_{600\text{ nm}}$ = 0.1) in the PBS buffer, in the presence or absence of glucose (25 mM), and in the presence or absence of a pump inhibitor (25 μM orthovanadate) were acquired over time with 3 s time interval at room temperature using a fluorescence spectrometer (Perkin-Elmer, LS50B). The excitation and emission wavelengths were selected at 350 and 450 nm, respectively.

**Structural modeling of fusion BmrA-EGFP transporters**

The BmrA model was assembled using SWISS-MODEL, based upon existing homologous structural models of the 'closed' multidrug ABC transporter
Sav1866 and LmrA with up to 33.7% sequence identity (PDB-codes: 2onj, 2hyd, 1mv5) [84, 85]. The N- (24 amino acids) and C- (15 amino acids) terminal regions of this model were truncated, based on residues 269-829 of the template model Sav1866 (2hyd). The total energy of the complete model was reported as -16525.7 kJ/mol.

The EGFP model was assembled separately using SWISS-MODEL, based on 3D structure of the GFP (PDB-code: 3ogo) with a sequence identity of 98.7%. The structure was truncated by 7 amino acids at the N-terminal and 3 amino acids at the C-terminal.

To predict the secondary structures of the truncated regions connecting the EGFP domain to BmrA, the complete protein sequences of the fusion proteins were analyzed using the PSIPRED server [86]. The results (Fig. 11A-B) predicted the linker regions to be unstructured and thus conformationally flexible. The flexible linker regions were built manually and docked to the homology model termini using program Coot [91]. The EGFP domains were positioned as such that they were situated in the cytoplasmic space with no clashes to the ABC transporter. The final models (Fig. 11C-D) were visualized using PyMOL [92].

Note: All experiments in this dissertation were conducted in compliance with the university safety guidelines. The IBC approval number for recombinant DNA research in chapter II & IV is 12-008.
CHAPTER III
PROBING OF MULTIDRUG ABC MEMBRANE TRANSPORTERS OF SINGLE LIVING CELLS USING SINGLE PLASMONIC NANOPARTICLE OPTICAL PROBES

INTRODUCTION

ATP-binding cassette (ABC) membrane transporters (efflux pumps) have been found in all living organisms, including bacteria and humans [52, 53]. For example, 78 ABC transporters have been reported in B. subtilis, and 48 ABC transporters have been identified in humans [52, 53, 55, 56]. Some of these transporters are involved in transport of a wide variety of structurally and functionally unrelated substrates (e.g., sugars, lipids, amino-acids, proteins, or xenobiotics), including extrusion of antibiotics or chemotherapeutic agents out of cells (e.g., bacteria or tumor cells). For example, P-glycoprotein accounts for multidrug resistance (MDR) in cancer cells, leading to ineffective treatments. Dysfunction of some of these ABC transporters causes severe diseases [9-11]. Despite extensive studies, such important and fascinating multi-substrate extrusion machinery and translocation mechanisms remain not yet fully understood [9, 52, 53].

Interestingly, all ABC transporters in any living organism share a common organization, possessing four core domains: two transmembrane domains (TMD) and two nucleotide-binding domains (NBD) [46, 59, 61, 93]. The TMDs display quite variable sequence and topology, defining the substrate binding-sites and
forming the transport passage-way for substrates to cross the membranes, while the NBDs have conserved sequences, empowering the transporters by binding and hydrolyzing ATP [46, 57, 61, 62]. The similar structures and organization of ABC transporters suggest possible similar transport mechanisms.

*B. subtilis* (a Gram-positive bacterium) is a well-studied bacterium with a full-sequenced genome, and it is amenable to genetic manipulation [80]. Therefore, *B. subtilis* has been widely used as a model organism for probing the function of prokaryotes and for better understanding of bacterial pathogenic functions (e.g., sporulation) and efflux function of multidrug membrane transporters, even though *B. subtilis* itself is not considered as a human pathogen [94, 95].

Currently, radioactively labeled substrates (\(^{14}\)C and \(^{3}\)H) and the fluorescence dyes (e.g., rhodamine 123, Fluo-3, Hoechst dyes) are common probes for investigating efflux kinetics of multidrug transporters in bacterial and mammal cells [68, 69, 74, 76]. Fluorescence dyes, such as Hoechst dyes and ethidium bromide (EtBr), are quinolones, common substrates for multidrug membrane transporters in bacteria. The fluorescence dyes emit weak fluorescence in aqueous solution (outside cells) and become strongly fluorescent in non-polar and hydrophobic environments, especially as the dye molecules enter cells and intercalate with DNA [77]. Therefore, the time-dependent (time-course) fluorescence intensity of fluorescence dyes has been widely used to characterize efflux function of multidrug membrane transporters in bacterial and mammal cells [68, 69, 74, 76].
Although these conventional methods effectively provide ensemble accumulation kinetics of bulk cells, they are unable to determine transformation of pore sizes of ABC membrane transporters in response to various sizes of substrates in single living cells. Therefore, it is impossible to use them for probing size-dependent efflux kinetics of multidrug (multi-substrate) ABC membrane transporters for better understanding of their functions and molecular mechanisms. These limitations demand the development of new methods for probing the size-transformation of individual membrane transporters in single living cells in real time, in order to better understand molecular mechanisms of multidrug membrane transporters. Individual membrane transporters are likely to have unsynchronized membrane transport kinetics, underscoring the importance of probing the transport kinetics of single membrane transporters in single living cells in real time.

Currently, the sizes of membrane transporters are characterized at atomic resolution using X-ray crystallography or cryo-TEM [61, 93, 96]. However, neither methods can measure real-time transport dynamics of substrates and self-assembly of pump proteins in living cells. Therefore, the mechanisms and functions of membrane transporters remain elusive, even though structures of some membrane transporters at atomic resolution are now available [61, 93, 96].

Noble metal (e.g., Ag) nanoparticles (NPs) exhibit size-dependent optical properties, enabling us to image and determine the sizes of single NPs at nanometer (nm) scale in real time using dark-field optical microscopy and spectroscopy (DFOMS) [42-44, 73, 75, 90]. Notably, the size-dependent
localized surface plasmon resonance (LSPR) spectra (colors) of single NPs enable us to image the sizes of NPs in solution, single living cells and embryos at nm resolution in real time [39, 43, 44, 75, 89, 90, 97, 98]. These unique features allow us to use the color (LSPR spectra) index of the multicolor NPs as a nanometer-size index to directly measure sizes of single NPs as they are transported in and out of single living cells, and thereby to determine the sizes of membrane pores at the nm resolution in real-time [75, 89, 90]. Unlike fluorescent probes and semiconductor quantum dots (QDs), these noble metal NPs resist photodecomposition and blinking and show superior photostability [43, 44, 97], enabling them to serve as optical sized probes for tracking the transformation of pore sizes of membrane transporters and the size-dependent transport kinetics of efflux pumps for any desired period of time.

We have previously used the intrinsic optical properties of the noble metal NPs (e.g., Ag) for probing of sizes and transport dynamics of a single membrane transporter (MexA,B-OprM) in single living cells (P. aeruginosa, Gram-negative bacterium), and the transformation of pore sizes of their cellular membranes induced by antibiotics (e.g., aztreonam, chloramphenicol) at sub-100 nm spatial resolution and millisecond temporal resolution [75, 89, 90].

In this study, we synthesized and characterized stable and purified spherical Ag NPs with an average diameter of $11.8 \pm 2.6 \text{ nm (8.4-31.5 nm)}$. We used their sized-dependent LSPR spectra to determine the sizes of single NPs at nm resolution in real time, when they were in and out of single living cells, which enabled us to probe size-dependent efflux kinetics of BmrA (one of ABC
membrane transporters) of single living bacterial cells (*B. subtilis*, Gram-positive bacterium). We also used the Ag NP probes to characterize BmrA fused with EGFP via its C- or N- terminus (Ct-BmrA-EGFP, or Nt-BmrA-EGFP) and to compare them with BmrA, aiming to determine whether the fused BmrA-EGFP maintains the efflux function of BmrA. We need to first determine whether BmrA-EGFP maintains the original efflux functions of BmrA, before we can use fused EGFP to visualize the BmrA locations and to characterize BmrA topologies, as those reported for other ABC transporters [65-67, 81].

**RESULTS AND DISCUSSION**

**Synthesis and characterization of single Ag NP optical probes**

We have synthesized spherical shaped Ag NPs and purified them by washing the NPs with nanopure deionized (DI) water using centrifugation. This approach enables us to remove any residual chemicals from the synthesis and to produce highly purified NPs. We found that the purified Ag NPs were stable in DI water for months, as we reported previously [44]. In order to maintain the viability of bacterial cells (*B. subtilis*), the cells must be suspended and incubated in the cell culture media or PBS buffer (0.5 mM phosphate buffered saline, 1.5 mM NaCl). Notably, the studies of accumulation and efflux kinetics of multidrug membrane transporters (efflux pumps) in bacterial cells (Gram-negative or Gram-positive bacteria) using fluorescence molecules have been carried out in the buffer solution (PBS buffer), but not in the cell culture medium [54, 68, 71-73, 99].
Given concentrations and stages of the cells (e.g., $OD_{600\,nm} = 0.1$) must be incubated with given concentrations of fluorescence molecules (substrates), in order to determine the dependence of accumulation and efflux kinetics of membrane transporters on substrate concentrations and on the expression level of membrane transporters in given cells. Note that the cells grow and divide in the medium, leading to the change of cell concentrations and thereby impossible comparison of the results among the experiments. Therefore, the cells are typically harvested from cell culture media, well rinsed and dispersed in the PBS buffer with given concentrations (e.g., $OD_{600\,nm} = 0.1$) for probing of transport kinetics of membrane transporters in living cells [54, 68, 71-73, 99].

It is crucial to ensure that single Ag NPs are stable (non-aggregated) in the PBS buffer over time, in order to utilize the size-dependent LSPR spectra (colors) of single NPs to determine their sizes, to probe the pore sizes of membrane transporters and to study size-dependent and concentration-dependent efflux kinetics of single membrane transporters of single living cells in the buffer.

We characterized the sizes, shapes and LSPR spectra of purified Ag NPs in the buffer for 24 h at single-NP resolution using high-resolution transmission electron microscopy (HRTEM) and dark-field optical microscopy and spectroscopy (DFOMS), respectively. This approach enabled us to study the stability (non-aggregation) of purified Ag NPs in the buffer for 24 h at single-NP resolution. The results in Fig. 12A and B show single spherical NPs with an average diameter of $12.1 \pm 4.8$ nm, ranging from 8.4 to 31.5 nm. The
representative optical images of single NPs in Fig. 12C show that the majority of NPs are plasmonic blue NPs with some being green and few red NPs. Histograms of color (size) distribution of single NPs (Fig. 12D) from 20 images similar to that in Fig. 12C, show 74% blue, 22% green and 4% red NPs (Fig. 12D-a), and 71% blue, 24% green and 5% red NPs (Fig 12D-b), before and after the NPs were dispersed in the buffer for 24 h, respectively.

We correlated the size distribution of single NPs measured by HRTEM (Fig. 12B) with the color (peak-wavelength of LSPR spectra) distribution of single NPs characterized using DFOMS (Fig. 12D), and found that blue, green and red NPs were correlated with NPs with diameters of 5-14 nm, 14-20 nm, and 20-32 nm, respectively [39, 75]. Therefore, we used the LSPR spectra (colors) of single NPs to determine their sizes in real-time, and used them as sized-probes to characterize the transformation of pore sizes of membrane transporters in single living cells and to study the size-dependent transport kinetics of multidrug membrane transporters (BmrA) of single living cells.

We also characterized the sizes, optical properties and stability (non-aggregation) of bulk Ag NPs in the buffer for 24 h using UV-visible absorption spectroscopy and dynamic light scattering (DLS). The UV-visible absorption spectra of purified Ag NPs before and after their incubation in the buffer for 24 h show an unchanged peak absorbance of 1.047 at 392 nm (Fig. 12E). The size distributions of the Ag NPs before and after their incubation in the buffer for 24 h
Fig. 12 Characterization of sizes, shapes and plasmonic optical properties of single Ag NPs, and their stability (non-aggregation) in PBS buffer.

NPs dispersed in the PBS buffer for (a) 0 min and (b) 24 h. (A) Representative HRTEM image and (B) histogram of the size distribution of Ag NPs. (C) Representative dark-field optical image of single NPs and (D) histograms of color distribution of single NPs. (E) UV–visible absorption spectra of 1.4 nM Ag NPs show peak absorbance of (a) 1.047 at 392 nm and (b) 1.043 at 392 nm. (F) Histograms of size distribution of single NPs in solution measured using DLS show the average diameters of NPs at (a) 11.8 ± 2.6 nm and (b) 11.8 ± 2.8 nm. The scale bars are 10 nm in (A) and 10 μm in (C).
characterized using DLS show the same distribution range with the average diameters of NPs at $11.8 \pm 2.6$ nm and $11.8 \pm 2.8$ nm (Fig. 12F), respectively. The results indicate that the sizes of Ag NPs in the buffer remain unchanged for 24 h. Interestingly, unlike our previous observations [44], the diameters of NPs measured in solution by DLS were slightly smaller than those measured under vacuum using HRTEM, which may be attributed to the negligible portion of larger NPs (18-30 nm in diameter) for ensemble measurements of DLS. In this study, the NPs in the buffer solution were used to characterize the efflux function of the BmrA transporter in single living bacterial cells ($B. subtilis$). Therefore, the average diameter of NPs is the one ($11.8 \pm 2.6$ nm) measured in solution using DLS. Taken together, the results in Fig. 12 show that purified Ag NPs are stable in the buffer solution for 24 h.

Real-time probing of size-dependent efflux kinetics of BmrA using single Ag NPs

We used the size-dependent LSPR spectra (plasmonic properties) of single Ag NPs to probe the size-dependent efflux function of BmrA by incubating the Ag NPs with the living cells ($\text{OD}_{600\text{ nm}} = 0.1$) and tracking single NPs in and out of single living cells in real time.

The representative optical images of the cells with single intracellular and extracellular NPs in Fig. 13A and B show cross-sections of single rod-like bacterial cells. The size of single bacterial cells is about 2 $\mu$m in length and 0.5 $\mu$m in width while the thickness of its cellular membrane is about 9 nm. Notably,
the top and bottom membranes of single living cells are invisible under dark-field illumination, which demonstrates that the focal plane (depth of field at 190 nm) of dark-field microscope indeed allows us to image the thin-layer sections of single living bacterial cells and to visualize single NPs on the thin-layer section of single cells.

The dark-field microscope illumination needs to pass through the cellular membrane in order to irradiate intracellular NPs, and light scattering of intracellular NPs requires passage through the membrane in order to be imaged by the detector. The cellular membrane absorbs photons, leading to lower scattering intensity of intracellular NPs (dimmer). In contrast, the scattering intensity of the extracellular NPs on the membrane contains the scattering intensity of NPs and cellular membrane, leading to radiating imaging and higher scattering intensity. These features allow us to distinguish between the intracellular and extracellular NPs. The intracellular NPs are dimmer and more blurry than NPs in solution, as shown in Fig. 13A-a and B-a. In contrast, the extracellular NPs are radiating and much brighter than NPs in solution, as shown in Fig. 13A-b and B-b. Notably, we have validated these imaging approaches for determining intracellular and extracellular NPs by imaging ultra-thin sections of cells using TEM [75].

The representative LSPR spectra of single NPs in Fig. 13C show their peak wavelength ($\lambda_{\text{max}}$) at 481 (blue), 535 (green) and 649 nm (red) NPs. Using the LSPR spectra (colors) of single Ag NPs, we identified the Ag NPs over any other possible cellular debris and vesicles, which do not have plasmonic
properties and thereby appear white under dark-field illumination. Furthermore, size-dependent plasmonic spectra enable us to determine the sizes of single NPs in real-time using DFOMS via the calibration curves of the peak wavelength ($\lambda_{\text{max}}$) of LSPR spectra of single NPs versus their sizes, as we reported previously [39, 44, 75, 89, 90]. The sizes of single blue, green and red NPs in Fig. 13C are 13.3, 21.8, and 31.5 nm, respectively.

We incubated WT BmrA cells ($OD_{600\,nm} = 0.1$) with 1.4 nM Ag NPs and continuously imaged single NPs in and out of single living cells in real time. Snap shots from real-time videos in Fig. 14A and B show representative single blue and green NPs in and out of single living cells, respectively. The plots of single NPs in and out of single living cells over time (Fig. 14C) show that the blue NP stays inside the cell for 66 min, while the green NP stays inside the cell for 14 min. The LSPR spectra of the blue and green NPs, similar to the one in Fig. 13C, show the peak wavelength ($\lambda_{\text{max}}$) at 479 nm and 540 nm, respectively. We determined the sizes of single blue and green NPs as 13.0 and 22.6 nm, respectively, using the correlation of sizes of single NPs determined by HRTEM with the peak wavelength of LSPR spectra (colors) of single NPs measured using DFOMS, as we reported previously [39, 75]. The results show that the smaller NPs stay inside the cell longer than larger NPs, suggesting size-dependent efflux kinetics of BmrA and indicating that smaller NPs are more biocompatible with the cells and they may be more suitable carriers for drug delivery.
Fig. 13 Imaging of single intracellular and extracellular Ag NPs for single living bacterial cells using DFOMS.

(A) The representative optical image of the cells (BmrA, OD$_{600}$ nm = 0.1) incubated with 1.4 nM Ag NPs shows: (a) intracellular and (b) extracellular NPs, in dotted rectangles. (B) Zoom-in images of single cells illustrate (a) intracellular and (b) extracellular NPs. (C) LSPR spectra of single Ag NPs show peak wavelengths of representative blue, green, and red NPs at 481, 535, and 649 nm, respectively. The scale bars in (A) and (B) are 10 and 2 μm, respectively.
Taken together, plasmonic Ag NPs offer the feasibility for probing of size-dependent efflux kinetics of membrane transporters in single living cells in real time. Unlike fluorescence probes (e.g., Hoechst dyes), single Ag NPs possess size-dependent LSPR spectra and they are photostable (non-photodecomposition and non-blinking) [43, 44, 97]. These superior properties enable us to determine the sizes of single NPs in and out of single living cells in real-time, and use them to mimic various sizes of antibiotics (drugs) for probing efflux functions of multidrug membrane transporters.

Probing of dependence of accumulation rates of NPs on expression level of BmrA

To determine whether BmrA and BrmA-EGFP are responsible for extrusion of NPs out of living cells, we studied the dependence of accumulation kinetics of intracellular NPs upon the expression level of BmrA and BrmA-EGFP in bulk living cells at single living cell resolution. The results in Fig. 15A show that ΔBmrA (strain of deleted BmrA) cells accumulate the highest amount of the intracellular NPs over time and have the highest accumulation rate (slope of the plot) of intracellular NPs, while the Nt-BmrA-EGFP cells (over expression level of BmrA with its N-terminal fusion with EGFP) accumulate the lowest amount of intracellular NPs and show the lowest accumulation rate.
Fig. 14 Real-time probing of BmrA membrane transporter of single living cells using single Ag NPs by DFOMS.

Snapshots of sequential images of (A) a single blue NP (13.0 nm in diameter) in and out of single living BmrA cells at (a) 18.0, (b) 32.0, (c) 47.0, (d) 60.0, (e) 74.0, and (f) 83.0 min; and (B) a single green NP (22.6 nm in diameter) in and out of single live cells at (a) 30.0, (b) 33.0, (c) 35.0, (d) 38.0, (e) 41.0, and (f) 44.0 min. (C) Plots of imaging of in and out of single NPs in (A and B) versus time show the duration of single blue and green NPs staying inside the cells: (a) 66.0 and (b) 14.0 min, respectively.
Fig. 15 Study of the dependence of accumulation and efflux kinetics of single Ag NPs and fluorescent dye (Hoechst 33342) molecules on the expression level of BmrA.

(A) Plots of number of intracellular Ag NPs versus time and (B) plot of fluorescence intensity versus time for: (a) ΔBmrA, (b) Ct-BmrA-EGFP, (c) WT-BmrA, and (d) Nt-BmrA-EGFP cells, show that accumulation kinetics of intracellular NPs and fluorescence dye molecules in single living cells depend on cellular expression level of BmrA. In (A), the points with error bars represent the averages of experimental measurements with their standard deviations; the lines are added to guide the trend. At each point (every 20 min), 900 cells were analyzed.
Interestingly, the accumulation rate and amount of intracellular NPs in Nt-BmrA-EGFP cells are slightly lower than the WT (normal expression level of BmrA), while the accumulation rate in WT cells is slightly lower than Ct-BmrA-EGFP cells (over expression level of BmrA with its C-terminal fusion with EGFP). The results suggest that WT (BmrA), Nt-BmrA-EGFP and Ct-BmrA-EGFP cells extrude the intracellular NPs, leading to less accumulation of intracellular NPs than ΔBmrA cells. The results indicate that Nt-BmrA-EGFP and Ct-BmrA-EGFP maintain the efflux function of BmrA, and N-terminal fusion generates less steric effects on the efflux function of BmrA than C-terminal fusion.

The sizes of NPs are about ten times larger than conventional antibiotics. To determine whether the NPs are suitable substrates to mimic various sizes of antibiotics for probing the efflux functions of BmrA, we used a fluorescence probe (Hoechst 33342) to study the dependence of accumulation kinetics on the cellular expression level of BmrA for probing the efflux function of BmrA and BmrA-EGFP membrane transporters [54, 76]. The Hoechst dye is a well-known substrate of BmrA (ABC) membrane transporter and has been widely used for probing the accumulation and efflux function of BmrA. The Hoechst dye emits weak fluorescence in aqueous solution (outside the cells), and its fluorescence intensity increases substantially (up to 10-fold) as the dye molecules enter the cells and intercalate with DNA [77]. Therefore, one can use the fluorescence intensity of the Hoechst dye to monitor intracellular accumulation of dye molecules [54, 76]. By probing the dependence of the accumulated amount of
the intracellular dye molecules on the expression level of BmrA membrane transporters, we determined the efflux kinetics of BmrA and BmrA-EGFP.

The results in Fig. 15B show that ΔBmrA cells accumulate the largest amount of intracellular dye molecules with the highest rate of accumulation (slope of the curve), whereas Nt-BmrA-EGFP cells accumulate the lowest amount of intracellular dye molecules with the lowest rate of accumulation. Notably, Nt-BmrA-EGFP cells, which have slightly lower accumulation rate and lower amount of the intracellular dye molecules than the WT cells, which have slightly lower ones than Ct-BmrA-EGFP cells. The results suggest that WT (BmrA), Nt-BmrA-EGFP and Ct-BmrA-EGFP extrude the dye molecules and thereby they accumulate less amount of the intracellular dye molecules than ΔBmrA cells, demonstrating that N-terminal or C-terminal BmrA fusion with EGFP maintains the efflux function of BmrA, and N-terminal fusion has less effect on the efflux function of BmrA than C-terminal fusion. Therefore, Nt-BmrA-EGFP and Ct-BmrA-EGFP cells become two new strains of BmrA available for further characterization of locations, structures and functions of BmrA. The results in Fig. 15B are similar to those observed using single NP optical probes (Fig. 15A), suggesting that single NP optical probes are well suited substrates for probing the efflux function of multidrug membrane transporters.

Unlike fluorescence probes, Ag NPs offer size information about the membrane permeability and substrates of the membrane transporters. The NPs, with sizes ranging from 8.4-31.5 nm, can enter the cells and be extruded out of the cells by BmrA and BmrA-EGFP transporters. Specifically, endocytosis,
pinocytosis and exocytosis, which are widely reported in eukaryotes, do not exist in prokaryotes (bacterial cells). Therefore, these processes are not responsible for the transport of NPs in and out of the bacterial cells (B. subtilis).

**Study of effect of pump inhibitor on accumulation rates of NPs in single living cells**

To determine whether BmrA and BmrA-EGFP membrane transporters are specifically responsible for the efflux of NPs, we studied the accumulation kinetics of the NPs in BmrA (WT), ΔBmrA, Nt-BmrA-EGFP and Ct-BmrA-EGFP living cells in the presence of a pump (ATPase) inhibitor, orthovanadate [82, 83]. The results in Fig. 16A show the higher accumulation rate of the NPs in the cells (WT-BmrA) in the presence of orthovanadate (Fig. 16A-a) than in its absence (Fig. 16A-b). In contrast, no significant change of accumulation of NPs in ΔBmrA cells was observed in the presence of the inhibitor (Fig. 16B).

Similar to WT cells, higher accumulation of NPs in Ct-BmrA-EGFP and Nt-BmrA-EGFP cells was observed in the presence of the inhibitor than its absence (Fig. 16C and D, respectively). These results show that the inhibitor affects the efflux of NPs by Ct-BmrA-EGFP, Nt-BmrA-EGFP and WT-BmrA transporters, suggesting that BmrA and BmrA-EGFP membrane transporters are indeed responsible for the efflux of NPs out of the cells. These results demonstrate that the NPs are the substrates of BmrA, and they are suitable optical probes to study efflux function of BmrA membrane transporters in single living cells.
Study of concentration-dependent accumulation rates of NPs in single living cells

We also investigated the dependence of accumulation rates of NPs on their concentrations (doses), in order to determine molecular mechanisms of entry of NPs into single living cells, and to compare them with those observed for antibiotics. We used two concentrations of NPs (0.7 and 1.4 nM) and studied their accumulation rates in BmrA (WT), ΔBmrA, Ct-BmrA-EGFP, and Nt-BmrA-EGFP cells. The results in Fig. 17 show the dependence of the number of intracellular NPs on NP concentrations for all strains of cells and the number of intracellular NPs increases as the concentration of NPs increases, suggesting that passive diffusion may play a role in transport of extracellular NPs into the cells, similar to passive diffusion of antibiotics into the cells.

The lowest number of intracellular NPs and lowest accumulation rates are observed in Nt-BmrA-EGFP (Fig. 17D), while the highest number of intracellular NPs and highest accumulation rates are found in ΔBmrA (Fig. 17B). Notably, the number of intracellular NPs at the equilibrium time, and accumulation rates in BmrA (WT) and Ct-BmrA-EGFP cells are similar to those observed in Nt-BmrA-EGFP. The results suggest that ΔBmrA cells are unable to extrude the NPs out of the cells, because of the absence of BmrA transporter, which leads to the highest accumulation of intracellular NPs. In contrast, the over-expression of Nt-BmrA-EGFP transporter effectively extrudes the NPs out of the cells, leading to the lowest accumulation of intracellular NPs. BmrA (WT) and Ct-BmrA-EGFP transporters extrude NPs out of the cells (Fig. 17A and C), leading to the lower
Fig. 16 Probing of inhibitory effects of an inhibitor on accumulation and efflux kinetics of single Ag NPs for single living cells.

Plots of intracellular Ag NPs versus time: (A) BmrA, (B) ΔBmrA, (C) Ct-BmrA-EGFP, and (D) Nt-BmrA-EGFP, (a) in the absence (empty triangles) and (b) presence (filled triangles) of the inhibitor (25 μM, orthovanadate). The points with error bars represent the averages of three experimental measurements with their standard deviations; the lines are added to guide the trend. At each point (every 20 min), 900 cells were analyzed.
Fig. 17 Study of concentration dependent accumulation and efflux kinetics of single Ag NPs for single living cells.

Plots of intracellular Ag NPs versus time: (A) BmrA, (B) ΔBmrA, (C) Ct-BmrA-EGFP, and (D) Nt-BmrA-EGFP cells, incubated with (a) 1.4 (filled triangles) and (b) 0.7 nM (empty triangles) Ag NPs. The points with error bars represent the averages of three experimental measurements with their standard deviations; the lines are added to guide the trend. At each point (every 20 min), 900 cells were analyzed.
accumulation of intracellular NPs than ΔBmrA cells. Therefore, the accumulation rates of intracellular NPs in BmrA (WT) and Ct-BmrA-EGFP cells are similar to those observed in Nt-BmrA-EGFP. As observed in Figs. 15A and 16, the results in Fig. 17 also show the dependence of accumulation rates of intracellular NPs on the expression level of BmrA.

We summarize quantitative accumulation rates and equilibrium times of accumulation of intracellular NPs in single living cells (Figs. 16 and 17) in Table 2, which shows their dependence on the expression level of BmrA, concentrations of NPs, and presence of the inhibitor.

Characterization of viability of single cells

Studies have found that Ag NPs show dose-dependent inhibitory effects on bacterial and tumor cells [39, 100]. Therefore, it is crucial for us to characterize the viability of cells that had been incubated with Ag NPs for hours, ensuring that Ag NPs did not affect the function and viability of the cells, and we indeed studied the membrane transporters (BmrA, BmrA-EGFP) of single living cells.

The viability of cells (WT, BmrA-EGFP, ΔBmrA) incubated with NPs throughout the duration of the experiments was characterized using live/dead bacLight viability and counting assay. The assay determines the viability of cells by detecting both live and dead cells using SYTO9 nucleic acid stain and propidium iodide, respectively. Observation of the green fluorescence ($\lambda_{\text{max}} = 520$
nm) of SYTO9 in bacterial cells shows viable cells. In contrast, the display of red fluorescence ($\lambda_{\text{max}} = 610$ nm) of propidium iodide in bacterial cells indicates the

Table 2 Summary of accumulation rates and equilibrium times of single NPs in single living cells

<table>
<thead>
<tr>
<th>C$_{Ag}$ NPs (nM)</th>
<th>Inhibitor$^a$ (µM)</th>
<th>Accumulation Rate (NPs/min)</th>
<th>Equilibrium Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT</td>
<td>ΔBmrA</td>
</tr>
<tr>
<td>0.7</td>
<td>0</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>1.4</td>
<td>0</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>1.4</td>
<td>25</td>
<td>1.7</td>
<td>1.8</td>
</tr>
</tbody>
</table>

$^a$ Orthovanadate

$^b$ Ct-BmrA-EGFP: cells with the over-expression of C-terminus BmrA fused with EGFP

$^c$ Nt-BmrA-EGFP: cells with the over-expression of N-terminus BmrA fused with EGFP
Fig. 18 Characterization of the viability of single bacterial cells using live/dead bacLight viability and counting assay.

(A) Dark field optical image and (B) fluorescence image of single bacterial cells (WT BmrA), incubated with 1.4 nM Ag NPs over the duration of each experiment for 4 h, show that the cells with intracellular NPs (dotted rectangles) or without NPs emit the green fluorescence ($\lambda_{\text{max}} = 520$ nm) of SYTO9, indicating viable cells. (c) Plots of percentage of viable cells at (a) 0 and (b) 4 h, for BmrA, ΔBmrA, Ct-BmrA-EGFP, and Nt-BmrA-EGFP cells, show that 99–100% of the cells are alive. A minimum of 900 cells for each strain were assayed at each given time. The scale bars in (A) and (B) are 10 μm.
dead cells. Representative optical images of the cells (WT-BmrA) incubated in the PBS buffer with 1.4 nM Ag NPs for 4 h (Fig. 18A), show the cells with and without NPs. Their fluorescence images in Fig. 18B illustrate the green fluorescence cells, showing that the cells with and without NPs are alive. Similar phenomena are observed for Ct-BmrA-EGFP, Nt-BmrA-EGFP, and ΔBmrA cells.

By counting the number of live and dead cells, we determined the percentage of the viable cells by dividing the number of live cells with the total number of the cells. Note that, for each measurement, we assayed at least 900 cells for each strain. The plots of the percentage of viable cells at the beginning and end of experiments in Fig. 18C show that more than 99% of the cells for each strain (WT, Ct-BmrA-EGFP, Nt-BmrA-EGFP, and ΔBmrA) were alive and viability remained constant throughout the duration of the experiments, demonstrating that we indeed studied the membrane transporters of single living cells. The results show that the NPs at these selected concentrations (1.4 nM) are biocompatible to the cells, demonstrating that the NPs are well suited for probing of membrane transporters of single living cells in real time.

SUMMARY

In summary, we have prepared purified and stable (non-aggregation) Ag NPs (11.8 ± 2.6 nm). In the given doses (1.4 nM), they are biocompatible with the cells. We utilized the size-dependent LSPR spectra of single NPs to determine the sizes of single NPs in and out of single living cells (B. subtilis) at nanometer resolution in real time using DFOMS, and to probe size-dependent
efflux kinetics of single BmrA membrane transporters (Ct-BmrA-EGFP, Nt-BmrA-EGFP) in single living cells in real-time. The results show that Nt-BmrA-EGFP and Ct-BmrA-EGFP transporters maintain the efflux function of BmrA, offering two new strains for the future study of BmrA. We found that the smaller NPs resided longer inside the cells than larger NPs, suggesting the size-dependent efflux kinetics of BmrA and indicating that smaller NPs are more biocompatible with the cells. Furthermore, accumulation and efflux rates of NPs for single living cells are dependent on the expression level of BmrA, concentration of NPs, and a pump inhibitor (orthovanadate), similar to those observed using a well-known substrate (Hoechst dye) of BmrA transporter, suggesting that NPs are substrates of BmrA transporters and they enter the cells via passive diffusion. Taken together, this study demonstrates that single plasmonic Ag NP optical probes are superior to fluorescence probes for study of efflux kinetics of multidrug membrane transporters in single living cells. Single Ag NPs exhibit size-dependent LSPR spectra and photostability (non-blinking and non-photodecomposition), offering the feasibility of using them to mimic various sizes of antibiotics (drugs) for probing the size-dependent efflux kinetics of membrane transporters of single living cells at nm resolution in real-time.

METHODS

Reagents and cell lines

Live/dead bacLight viability and counting assay and Hoechst 33342 were purchased from Invitrogen; all other reagents were purchased from Sigma-
Aldrich, and used as received. For cell lines of *Bacillus subtilis*, WT (BmrA) were purchased from Bacillus Genome Stock Center (BGSC), while ΔBmrA (previously named ΔYvcC, a mutant strain that is void of the BmrA) were provided by J. M. Jault [54]. The cell lines of Ct-BmrA-EGFP (54A, a mutant with over expression level of BmrA with its C-terminal fusion with EGFP) and Nt-BmrA-EGFP (29A, a mutant with over expression level of BmrA with its N-terminal fusion with EGFP) were constructed in our laboratory. The design and characterization of Nt-BmrA-EGFP and Ct-BmrA-EGFP will be fully described and reported in elsewhere.

**Synthesis and characterization of Ag NP optical probes**

Silver NPs were synthesized and washed as we described previously [44, 97]. The purified Ag NPs were dispersed in the PBS buffer (0.5 mM phosphate buffer, 1.5 mM NaCl, pH = 7.0). We characterized the sizes, concentrations and number of single NPs in the buffer over time to determine the stability (non-aggregation) of NPs in the buffer. The sizes of single NPs were determined using high-resolution transmission electron microscopy (HRTEM) (FEI Tecnai G2 F30 FEG) and dynamic light scattering (DLS) (Nicomp 380ZLS particle sizing system). The concentrations of NPs were measured and calculated, as described previously [97, 101]. Plasmonic absorption and scattering properties of bulk NPs were characterized using UV-visible spectrometer (Hitachi).

The plasmonic images and LSPR spectra of single NPs were acquired using dark-field optical microscopy and spectroscopy (DFOMS) equipped with
CCD camera (Micromax, Roper Scientific) and Multiple-spectra imaging system (Nuance, CRI) [39, 43]. The design and construction of DFOMS have been fully described in our previous studies [37-39, 42, 43, 97, 98,]. The dark-field optical microscope was equipped with a dark-field condenser (Oil 1.43-1.20, Nikon) and a 100x objective (Nikon Plan fluor 100x oil, iris, SL. N.A. 0.5-1.3, W.D. 0.20 mm) with the depth of field (focus) of 190 nm.

Cell culture and preparation

We pre-cultured the cells in an Erlenmeyer flask (250 mL) containing 20 mL of L-broth (LB) medium (1% tryptone peptone, 0.5% yeast extract, and 0.5% NaCl, pH = 7.2) in a shaker (Lab-line Orbit Envivon-Shaker) (150 rpm, 37 °C) for 12 h. We then cultured the cells (WT and ΔBmrA) in the LB medium for another 8 h, and cultured the Ct-BmrA-EGFP and Nt-BmrA-EGFP cells in the medium containing 1% xylose for another 8 h to ensure the full expression of the EGFP. We harvested the cultured cells using centrifugation (Beckman Model J2-21 Centrifuge, JA-14 rotor, at 7500 rpm, 23 °C, 10 min), washed the cells with the PBS buffer three times, and finally re-suspended the cells in the buffer. The final concentration of the cells was adjusted to OD$_{600\text{nm}}$ = 0.1, and used for the entire study.

Imaging of single NPs in single living cells and probing cellular viability

The cell suspension (OD$_{600\text{nm}}$ = 0.1) containing 0.7 and 1.4 nM Ag NPs in the absence and presence of orthovanadate (25 μM) were prepared. The timer
was started to record the incubation time as the NPs were added into the cell suspension.

We transferred the mixture (20 µL) into a freshly prepared microchamber and continuously imaged and acquired LSPR spectra of single NPs in and out of single living cells in real time for 3 h using DFOMS, which enabled us to determine the sizes and positions of single NPs simultaneously. The construction of microchamber for imaging of single bacterial cells over time was fully described in our previous studies [37-39, 42, 43, 97, 98]. We could achieve the temporal resolution up to 5 ms with sufficient high signal to noise ratios for continuously imaging the transport of single NPs in and out of single living cells. However, we found that the transport of single NPs in and out of single living cells (*Bacillus subtilis*) was not a rapid process and a temporal resolution of seconds to two minutes was sufficient to track their transport in real time. This approach enabled us to avoid generating huge sizes (> 200 GB) of single data files that would be unnecessary and hard to handle and analyze.

We also sampled the mixture (20 µL) into a freshly prepared microchamber every 20 min and imaged the cells on ten representative locations in the microchamber using DFOMS equipped with a CCD camera. This approach enabled us to image massive amount of cells (3000 cells) for each sample to gain sufficient statistics for probing the accumulation rates of bulk cells at single cell resolution. We determined intracellular NPs and plotted them versus time to measure the accumulation rates of single NPs in the cells over time (accumulation rate = slope of the plot).
At the end of each experiment, the viability of the cells was characterized using live/dead BacLight bacterial viability and counting assay [102]. We imaged the cells using dark-field optical microscopy and epi-fluorescence microscopy, and counted the green fluorescence cells (peak wavelength of fluorescence spectra of SYTO9, $\lambda_{\text{max}} = 520$ nm) and red fluorescence cells (peak wavelength of fluorescence spectra of propidium iodide, $\lambda_{\text{max}} = 610$ nm) as live and dead cells, respectively.

**Fluorescence spectroscopy measurements**

Time-dependent (time-course) fluorescence intensity of Hoechst 33342 (2 $\mu$M) incubated with the cells (OD$_{600}$ nm = 0.1) was measured at a 3-s data acquisition interval in real-time using a fluorescence spectrometer (Perkin-Elmer LS50B). The excitation and emission wavelengths were 350 and 455 nm, respectively.

**Data analysis and statistics**

For characterization of sizes, shapes, and LSPR spectra of single NPs using HRTEM and DFOMS, a minimal of 100 Ag NPs were imaged for each measurement. Each measurement was repeated three times. Thus, 300 NPs were characterized for each solution.

We acquired ~30 cells in a single CCD image in real time simultaneously. Therefore, we studied the transport of single NPs in and out of ~30 of single living cells for each measurement. We repeated the experiment three times and
hence ~90 cells were studied to determine the transport of single NPs in and out of single living cells in real time.

We also acquired ten representative images of each cell suspension incubated with 0.7 or 1.4 nM Ag NPs every 20 min for 3 h to study accumulation rates of intracellular NPs in single living cells in the presence or absence of the inhibitor (orthovanadate, 25 μM). Therefore, a minimal of ~300 cells was imaged every 20 min, and 2700 cells were studied over 3 h for each measurement. Each experiment was repeated three times. Thus, 8100 cells were studied for each sample, which allowed us to gain sufficient statistics for probing accumulation rates and efflux kinetics of bulk cells at single cell resolution. We analyzed the number of intracellular NPs in 900 cells (300 cells for each measurement) at each 20 min, plotted them over time, and used the slope of the plots to determine the accumulation rates of intracellular NPs in the cells in the presence and absence of the pump inhibitor (orthovanadate, 25 μM). The equilibrium times of accumulation of NPs in the cells were determined at the times when the accumulation rates of intracellular NPs remained unchanged over time. The amount of accumulated intracellular NPs for each strain refers to the amount of intracellular NPs at the equilibrium time.

For the study of viability of single cells, a minimal of 300 cells incubated with 1.4 nM Ag NPs for 12 h was assayed for each measurement. Each measurement was repeated three times. Thus, 900 cells were assayed for each sample.

Note: The following guidelines from the University of Oklahoma Health
Sciences Center were always followed whenever we are working with nanoparticles involved in the research studies shown in chapter III & V (http://www.ouhsc.edu/ibc/documents/NanoparticlesGuidelines.doc).

1. Wear lab coats at all times.

2. Wear gloves when handling nanoparticles and gloves must cover the hand and the wrist.

3. Wear long arm sleeves where high levels of exposure to nanoparticles were anticipated.


5. Perform work in a chemical hood or biological safety hood.
   a. Never conduct aerosol producing activities (such as sonication, vortexing, or centrifuging) on an open lab bench.
   b. Do not perform activities that could possibly release nanoparticles (such as emptying and opening of tubes) on an open lab bench.
   c. Hand solutions containing nanoparticles over disposable lab bench covers.

6. Provide hand washing facilities in all labs. Wash hand after handling nanoparticles.

7. Clean lab bench tops and other surfaces after each experiment using a methanol solution appropriate for the particular type of nanoparticles being used.
CHAPTER IV

STUDY OF STRUCTURE AND EFFLUX FUNCTION OF EGFP FUSED MEXA,B-OPRM MEMBRANE TRANSPORTER IN PSEUDOMONAS AERUGINOSA

INTRODUCTION

MDR (Multidrug resistance) associated efflux pumps have been identified in many organisms, ranging from bacteria to humans [2, 5, 8, 47]. In bacteria, the genes encoding for efflux pumps are usually located on the chromosome or transmissible genetic elements like plasmids and it is well known that the efflux pumps in bacteria confer lower susceptibility to antibiotics, however this does not necessarily result in increased antibiotic resistance which may be contributed by other causes than existence of efflux pumps [7, 103]. To date, five classes of efflux pumps are found associated with multidrug resistance: the MF superfamily, the SMR family, the multidrug and toxic-compound extrusion (MATE) family, the ABC superfamily and the RND family. Those efflux pumps are different in the number of assembled components, transmembrane domains, the energy sources used by pumps as well as the types of substrates exported by pumps [2, 3, 5, 8, 46]. Among these MDR transporters, only the ABC efflux transporters are primary transporters with the rest being secondary transporters utilizing sodium or proton gradient as the source of energy, whereas the MF superfamily dominates in gram-positive bacteria, the RND family only exists in gram-negative bacteria [2, 5, 8, 103]. Besides from exporting multiple antibiotics out of cells,
those efflux pumps are found to play new roles recently such that some efflux pumps of the resistance-nodulation-cell division (RND) family have been identified to have a role in the survival and colonization of the bacteria inside the host. In addition, other efflux pumps are found to be capable of exporting natural substances produced by the host [104-106].

Efflux pumps of RND family (e.g., MexA,B-OprM) which are expressed by gram-negative bacteria and are found to be responsible for multidrug resistance in clinical therapies are tripartite systems including the following three components: a transporter protein (e.g., MexB), which is located on the inner membrane of the bacterium; an outer-membrane protein (e.g., OprM), which is located in the outer-membrane of the bacterium and a membrane fusion protein (e.g., MexA) which is located in the periplasmic space [4, 107]. Taking the MexA, B-OprM efflux pump as an example, it is generally thought that the transporter protein MexB captures its substrates either from within the lipid bilayer of inner membrane or from cytoplasmic contents, then transports them to the extracellular medium through OprM which forms a channel in the outer membrane [108]. The cooperation between MexB and OprM is coordinated by the periplasmic membrane fusion protein MexA [15, 109-112]. Efflux through RND-family pumps is driven by the proton motive forces, an electrochemical gradient resulted from the motion of hydrogen ions. Besides, the outer-membrane protein OprM of P. aeruginosa is found to interact with several other RND family proteins [7, 111].

Despite extensive studies, the translocation mechanisms of those transporters remain elusive [109-111, 113, 114]. Many fundamental questions
regarding molecular mechanisms and interactions of multidrug efflux transporters remain unanswered. For instance, the individual integrated steps of the catalytic cycle, which are responsible for transporting substrates out of the membrane, are still at large though a lot of researches in delineating the efflux mechanisms have been done in the past [45, 115-117]. Notably, fusing membrane transporters with EGFP will offer the opportunity to visualize them and thus to investigate their interactions with substrates that lead to their extrusions. However, the fusion of multi-drug efflux transporters with EGFP may disrupt their original efflux functions. Therefore, it is crucial to first confirm that these fusion proteins still maintain the original efflux functions prior to utilizing them to probe molecular mechanisms of multidrug efflux pumps.

The past two decades have witnessed numerous uses of fluorescent protein (e.g., GFP or its variants) in the study of localization and expression of proteins in all kinds of organisms [23, 118-120]. For example, fusing promoter region with fluorescent proteins has been used to identify the multidrug efflux pump genes in Caenorhabditis elegans and to visualize the locations of MDR transporters in fission yeast [24, 64]. Besides, GFP has also been used as a fluorescence probe to characterize the topologies of multidrug efflux transporters, because GFP only fluoresces within the cytoplasmic space (reducing environment), but not in the periplasm or plasma area [65]. Studies have shown that GFP fusion proteins may alter the function of proteins. Unfortunately, the lacking of the systematic studies on these areas is often the case [121].
Functional studies of multidrug efflux pumps in bacterial and eukaryotic cells often rely on measuring of the accumulation of radioactively labeled substrates ($^{14}$C and $^{3}$H) or the fluorescence dyes (e.g., rhodamine 123, Fluo-3, Hoechst dyes) [68, 70]. Fluorescent dyes, such as Hoechst dyes and ethidium bromide (EtBr), are particularly suitable for the study of the MDR mechanisms because of the big increase of fluorescence intensities when they are changed from aqueous solutions to non-polar and hydrophobic environments, especially when the dye molecules intercalate with DNA. Therefore, change of time-dependent fluorescence intensity of fluorescence dyes will reflect the real-time state of the accumulation of substrates in bacterial and eukaryotic cells [71, 99].

Efflux functions of multidrug efflux pumps have also been characterized by determining the minimal inhibitory concentrations (MICs) of antibiotics. These antibiotics are specific substrates of these transporters and different strains have demonstrated the different susceptibility towards them. For example, a wide range of antibiotics such as chloramphenicol (CP), tetracycline (TET), gentamicin (GM), aztreonam (AZT), rifampicin (RIF), ofloxacin (OFLX) and novobiocin (NOV) have been utilized to detect the efflux functions of MexA,B-OprM efflux pump and different *P. aeruginosa* strains showed different MICs of the selected antibiotics [13, 116].

In this study, we constructed the *EGFP-mexB* fusion gene and successfully expressed it in ΔMexB strain of *Pseudomonas aeruginosa*, thus generating a new strain of *Pseudomonas aeruginosa* (MexA-EGFP-MexB-OprM). To determine whether EGFP-MexB subunit can coordinate with other two pump
components-MexA and OprM to form a complete efflux pump and retain its efflux functions, we characterized the real-time extrusion of fluorescence dye molecules (EtBr) by these strains in the presence or absence of a MDR pump inhibitor—CCCP using fluorescence spectroscopy. We also determined the minimal inhibitory concentrations (MICs) of selected antibiotics such as AZT and CP for these strains. To our knowledge, fusion of EGFP with MexB transporter in *Pseudomonas aeruginosa* has not yet been reported and a functional EGFP fusion strain can be used to study functions and structures of MexA,B-OprM transporter in single living cells.

**RESULTS AND DISCUSSION**

**Construction and characterization of EGFP-mexB fusion gene and its expression**

We designed and constructed the *EGFP-mexB* fusion gene by fusing *EGFP* gene to the N-terminal of *mexB* gene and transformed the plasmid containing fusion gene into the ΔMexB strain to create a new strain of *Pseudomonas aeruginosa* (MexA-EGFP-MexB-OprM). The fusion plasmid, pMMB67EH-EGFP-MexB (67PB), is constructed as shown in Fig. 19 with details as described in the experimental section. Briefly, the genomic DNA of *Pseudomonas aeruginosa* was used to amplify *mexB* gene by a set of primers (*mexB* P1 and *mexB* P2) while *EGFP* gene was amplified from pEGFP vector via another set of primers (*egfp* P1 and *egfp* P2). Amplified *EGFP* and *mexB* PCR products were then subject to a secondary round of PCR amplification by a set of
primers (egfp P1 and mexB P2). Then the PCR products of EGFP-mexB fusion gene and vector (pMMB67EH) were then digested using a pair of restriction endonuclease enzymes (SalI/HindIII). The digested PCR products (3,750 base pairs, bp, EGFP-mexB gene) were finally ligated with the digested and purified vector (8,800 bp), pMMB67EH. This approach enables us to insert EGFP-mexB fusion gene into the vector to create the fusion plasmid (pMMB67EH-EGFP-MexB, 67PB).

The amplified PCR products were characterized using routine agarose gel electrophoresis and DNA sequencing. The results in Fig. 20A show that amplified PCR products of mexB and EGFP are about 3,000 bp and 750 bp, respectively, which agree well with the number of base pairs of the mexB and EGFP genes. The fusion plasmid (pMMB67EH-EGFP-MexB, named as 67PB by us) in Fig. 19 was characterized using DNA agarose gel electrophoresis and DNA primer walking sequencing. The fusion plasmid digested by SalI/HindIII (Fig. 20B) showed two products of about 8,000 bp and 4,000 bp, which comply with expected sizes of digested vector and EGFP-mexB fusion gene, respectively. The DNA sequencing results of the fusion gene show that the sequences of the mexB gene are identical with those reported previously [14, 15, 116, 122] and that the EGFP gene is in same open reading form (ORF) with mexB gene without any linking sequence between them.
Fig. 19 Schematic illustrations of construction of \textit{EGFP-mexB} fusion gene.

(A) The double and single stranded \textit{EGFP} gene (step I-II) was amplified from plasmid pEGFP by a set of primers (\textit{egfp} P1 and P2) and a single primer (\textit{egfp} P1), respectively. (B) The genomic DNA from wide type PA01 strain was used to amplify double or single stranded \textit{mexB} gene via a pair of primers (\textit{mexB} P1 and P2) and a single primer (\textit{mexB} P2) (step I-II), respectively. The amplified products were further mixed and amplified by \textit{egfp} P1 and \textit{mexB} P2 primers through asymmetrical PCR to get the fusion gene \textit{EGFP-mexB} (A-B, step III). (C) The fusion gene and vector (pMMB67EH) were digested by restriction enzymes (\textit{SalI}/\textit{HindIII}). The digested fusion gene products were then ligated with the digested vector, generating the fusion plasmid, pMMB67EH-EGFP-MexB (IV)
Fig. 20 Characterization of *EGFP-mexB* fusion gene using agarose gel electrophoresis.

A-L1: DNA markers/ladders in base pair (bp); L2: *EGFP-mexB* fusion gene amplified using the mix of amplified *EGFP* and *mexB* gene products as a template and *egfp* P1 and *mexB* P2 as primers; L3: *mexB* gene amplified using the genomic DNA of *P. aeruginosa* strain (PA01) as a template and *mexB* P1 and P2 as primers; L4: *EGFP* gene amplified using the plasmid (pEGFP) as a template and *egfp* P1 and P2 as primers. Arrows point to the PCR products of about 750, 3,000 and 3,800 bp, which agree well with the number of base pairs of *EGFP*, *mexB* and *EGFP-mexB* genes. B-L1: DNA markers in bp; L2: pMIMB67EH-EGFP-MexB plasmids are digested by a pair of restriction endonuclease enzymes, *SalI/HindIII*. Arrows point to the digested vector plasmids of about 8,800 bp and *EGFP-mexB* fusion gene of about 3,800 bp.
Then we transformed the fusion plasmid (pMMB67EH-EGFP-MexB, 67PB) into the ΔMexB strain (the strain with deletion of the mexB gene) to create a new strain of *Pseudomonas aeruginosa* (MexA-EGFP-MexB-OprM), and characterized its expression in single living cells by measuring fluorescence of EGFP in single living cells using fluorescence microscopy and spectroscopy. The results in Fig. 21 show that nearly 100% of the MexA-EGFP-MexB-OprM cells emit green fluorescence, but none of the wild-type strain (PA01), the efflux pump overexpressed strain (NalB) and the pump deleted strain such as ΔMexB and ΔMexA,B-OprM emit green fluorescence, demonstrating that the fusion gene are successfully transformed and expressed in the ΔMexB cells.

Thus, the new strain enables characterization of locations, topologies and functions of MexB component of MexA, B-OprM efflux pump in living cells, as those reported for other MDR transporters [65]. However, in order to use them to study the functions and structures of MexB proteins, we have to determine first if EGFP-MexB plus MexA and OprM components can form a functional extrusion pump.

**Probing of dependence of efflux kinetics of transporters on their expression and the presence of pump inhibitor in live cells**

Except from its use to label DNA fragment in molecular biological experiments, EtBr is also a well-known substrate of MDR transporters (e.g., MexA,B-OprM) and has been widely used to monitor the accumulation and efflux kinetics of these transporters [70, 99, 123, 124]. Notably, the EtBr dye molecules
emit weak fluorescence in aqueous solution (outside the cells), and their fluorescence intensity increases substantially (up to 10-fold), as they permeate into the cells and intercalate with DNA. Therefore, change of the fluorescence intensity of the EtBr dye molecules has reflected the real-time accumulation and efflux kinetics of multidrug efflux pumps in living cells.

Fig. 21 Characterization of expression of EGFP-mexB fusion gene in P. aeruginosa.

(A) Dark-field optical images and (B) green fluorescence images of single living cells: (a) PAO1; (b) NalB; (c) ΔMexA,B-OprM; (d) ΔMexB and (e) MexA-EGFP-MexB-OprM strains, respectively. Scale bar = 5 μm
To determine whether EGFP-MexB protein expressed in the ΔMexB cells can form a functional efflux pump with other components as MexA and OprM, we first studied the efflux kinetics of the EtBr dyes by those different *P. aeruginosa* strains containing different expression level of MexA,B-OprM pump proteins in living cells in real time using the fluorescence spectroscopy.

We measured the time-dependent fluorescence intensity of the EtBr dye molecules (10 and 40 μM) incubated with living cells (ΔMexB, MexA-EGFP-MexB-OprM, PA01, NalB and ΔMexA,B-OprM; OD_{600 nm} = 0.1) in PBS (pH=7.2) over time. The results in Fig. 22 show that, the fluorescence intensity of the dye molecules increases over time, indicating that the dye molecules enter the cells and intercalate with intracellular DNA. Notably, the accumulation rates highly depend upon the expression level of MexA,B-OprM pump proteins. The results in Fig. 22A and B all show that the highest accumulation rate of the intracellular dye molecules with the highest fluorescence intensity was found in ΔMexA,B-OprM cells (deletion of MexA,B-OprM), while the lowest accumulation rate was observed in NalB cells (MexA,B-OprM pump overexpressed strain), regardless of the concentration of EtBr dyes used. The MexA-EGFP-MexB-OprM fusion strain only shows a slightly lower accumulation rate of dye molecules than the ΔMexB strain (deletion of MexB), suggesting that fusion of MexB with EGFP fails to restore the efflux function of MexB antiporter fully. This could be due to steric hindrance effects of fusing EGFP with MexB gene. In addition, the accumulation rate of dye molecules in the PA01 strain (Normal expression of MexA,B-OprM) is lower than that in the fusion strain (MexA-EGFP-MexB-OprM).
Fig. 22 Study of the dependence of efflux kinetics of the intracellular dye molecules on the expression of MexA,B-OprM transporter in live cells.

Time-dependent fluorescence intensity of different concentrations (A-10µM, B-40 µM) of EtBr dye molecules incubated with cells (OD$_{600}$ nm = 0.1 in PBS, pH 7.2) over time: (a) ΔMexA,B-OprM, (b) ΔMexB, (c) MexA-EGFP-MexB-OprM (d) PAO1 and (e) NalB strains
Interestingly, the WT PAO1 strain shows a slightly lower accumulation rate than the ΔMexB or fusion strain (MexA-EGFP-MexB-OprM) does, especially in the presence of 10μM EtBr dyes and this leads us to question whether it is enough to probe the function of MexA,B-OprM efflux pump just by monitoring the accumulation rates of EtBr dye molecules. The results we observed in this study might be due to the interplay of OprM with other MDR pump components such as MexX, MexY to form another MDR transporter (MexX,Y-OprM). Besides, other MDR pumps like MexC,D-OprJ, MexE,F-OprN and MexH,I-OprD in *Pseudomonas aeruginosa* could also extrude EtBr dyes [5, 47].

In addition, we also studied the effects of a PMF (proton motive force) inhibitor, CCCP, on the accumulation of intracellular EtBr dye molecules. The results in Fig. 23 demonstrated that in the presence of 0.1mM CCCP [125], all strains have increased accumulation of EtBr dye molecules inside cells. This indicated that CCCP can inhibit the efflux function of MexA,B-OprM pump. But, the inhibitory effects are not specifically on this pump since the accumulation of EtBr dyes in ΔMexA,B-OprM strain also increased in the presence of CCCP. This could be due to the inhibitory effects of CCCP on other MDR pumps using PMF as energy sources such as MexC,D-OprJ and MexE,F-OprN.

**Minimum inhibitory concentrations (MICs) of antibiotics for different *Pseudomonas aeruginosa* strains**

*P. aeruginosa* has been reported to have intrinsic antibiotic resistance against a wide range of antibiotics such as chloramphenicol (CP), tetracycline
Fig. 23 Probing of inhibitory effects of a pump inhibitor (CCCP) on efflux function of MexA,B-OprM and MexA-EGFP-MexB-OprM transporters in live cells.

Time-dependent fluorescence intensity of the dye molecules (EtBr; 15 μM), incubated with cells (OD$_{600}$ nm = 0.1 in PBS, pH 7.2): (A) ΔMexA,B-OprM, (B) ΔMexB, (C) MexA-EGFP-MexB-OprM, (D) PA01 and (E) NalB, in presence (a) and absence (b) of CCCP (0.1 mM)
(TET), azthreonam (AZT), gentamicin (GM) [5, 17, 112, 126, 127].

To further access the efflux function of the fusion strain (MexA-EGFP-MexB-OprM), we determined the MICs of two sensitive antibiotics (CP and AZT) for five mutant strains. The results in Table 3 indicate that the fusion strain partially restored the efflux function of MexA,B-OprM pump as is shown by the very small difference between the MICs of AZT and CP in the mutant lacking MexB (MIC of AZT- 0.2 µg/mL and MIC of CP- 4 µg/mL) and those in the EGFP fusion strain (MexA-EGFP-MexB-OprM; MIC of AZT- 0.5 µg/mL and MIC of CP-8 µg/mL). However, there is a substantial difference between the MICs of AZT and CP in the EGFP fusion strain and those in the PA01 or NalB strain. For instance, MICs of AZT and CP in the PA01 strain are 25 and 3.13 µg/mL, respectively, while those in the NalB strain are 100 and 15.7 µg/mL, respectively.

<table>
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<tr>
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**Structural modeling of the MexA-EGFP-MexB-OprM multidrug efflux pump**

Initial coordinates of the complete trimeric pump were obtained from the data-driven docking of AcrA-TolC onto AcrB [128]. The individual components of
the tripartite multidrug efflux pump from *P. aeruginosa* are highly homologous to
the corresponding components in the MexA,B-OprM transporter system with up
to 69 % sequence identity between the antiporter AcrB and MexB [84]. Three
dimensional structure alignment showed low average RMSDs for coordinate
displacements of 2.053 Å for AcrA/MexA (PDB codes: 2F1M and 1VF7), 2.355 Å
for AcrB/MexB (PDB codes: 2J8S and 2V50) and 2.064 Å for TolC/OprM (PDB
codes: 2VDD and 1WP1) [129, 130]. The working model was assembled by
secondary structure matching of MexB on AcrB in its trimeric configuration [91,
130]. The EGFP model was assembled separately using SWISS-MODEL [85],
based on 3D structure of the GFP (PDB code: 3OGO) with a sequence identity of
98.7%. Secondary structures prediction [86] showed the linker region connecting
the C-terminus of the EGFP domain to the N-terminus of MexB to be
unstructured. The connecting residues were built manually using program Coot
[91]. The EGFP domains were positioned in the cytoplasmic space with no
clashes to the MexB domains and the membrane region. All Figures (Fig. 24A-B)
were generated using PyMOL [92].

Fig. 24A shows the assembled MexA-MexB-OprM model based on the
coordinates of the data-driven docking model of the multidrug efflux pump AcrA-
AcrB-TolC from *P. aeruginosa* [128]. The model shows the location of the MexB
N-termini at the cytosolic end of the translocation pore of the transporter system
with the unstructured loop of the N-termini pointing inward towards the trimeric
core.
Fig. 24 Structural analysis of the assembled MexA-MexB-OprM and MexA-EGFP-MexB-OprM trimers.

(A) Bottom view of the assembled MexA-MexB-OprM trimer. The membrane
fusion protein MexA and the outer membrane channel OprM are shown in turquoise and violet, respectively. The individual antiporter MexB monomers are depicted in red, orange and light brown. Black O rings point to the position of the N-termini of the MexB monomers at the end of the N-terminal loop pointing towards the translocation pore of the trimeric antiporter; (B) Side (a) and bottom (b) view of the proposed model of the MexA-EGFP-MexB-OprM trimer

Fig. 24B shows the EGFP domains fused to the N-terminal of MexB in its trimer assemblage. Spatial placement of the EGFP domains in the cytosol is limited by potential EGFP to EGFP, or EGFP to MexB clashes in the trimeric configuration (Fig. 24B). The position and orientation of the N-terminal fused EGFP domains may either partially block the translocation pore, or restrict the movement of the individual pump domains thereby restricting efflux activity.

SUMMARY

In summary, we have designed and constructed a EGFP-mexB gene and successfully expressed the fusion gene in the ΔMexB strain to generate a new strain of Pseudomonas aeruginosa (MexA-EGFP-MexB-OprM). We studied the dependence of accumulation kinetics of intracellular fluorescence molecules (EtBr, a pump substrate) on the expression level of MexA,B-OprM pump proteins for each of the strains (PA01, NalB, ΔMexB, MexA-EGFP-MexB-OprM and ΔMexA,B-OprM), by measuring the time-dependent fluorescence intensity of the intracellular dye molecules using fluorescence spectroscopy. We found that
MexA-EGFP-MexB-OprM can extrude the pump substrate (EtBr). However, there is no big difference in terms of the accumulation rate of intracellular fluorescent dyes between the fusion strain and the ΔMexB strain. Besides, CCCP was shown to inhibit the efflux pumps in all the tested strains. Also Minimum Inhibitory Concentration (MIC) studies of two selected antibiotics in these two strains yielded outcomes of only about two times of differences. These results all suggest that the fusion strain partially restores the efflux function of MexA,B-OprM transporter, even though we observed the positive interaction between EGFP part of fusion protein with Ag nanoparticle based bio-sensor during the in vitro experiment (Data not shown). Putative structural analysis shows a possible obstruction of the EGFP part to the MexB one, resulting in conformational hindrance, which may obstruct MexB to coordinate with MexA and OprM to form a completely functional efflux pump.

Work is in progress to create the new strains for probing molecular mechanisms and structures of the MexA,B-OprM multidrug resistance efflux pump. Future work can be done on the fusion of GFP variants (RFP and CFP) to other two subunits (MexA and OprM). The Fluorescence Resonance Energy Transfer (FRET) study between either of two or three subunits of MexA,B-OprM efflux pump can be performed as well to study the interplay between them. Those future studies combined with utilization of nanoparticle based optical sensors to probe the efflux functions of this MDR transporter will provide more insights into its molecular mechanisms. However, it remains a challenge to get three fully functional fusion proteins [113, 114].
METHODS

Reagents and cell strains

PCR polymerase (Agilent Technologies), plasmid isolation kit (Qiagen), DNA gel extraction kit (Qiagen), DNA ligation kit (Roche) which includes ligation enzyme (T4 DNA ligase) and ligation buffer, SalI/HindIII (NEB), Tetracycline (Calbiochem), Chloramphenicol (Calbiochem), IPTG (GBT), CCCP (carbonyl cyanide-m-chlorophenylhydrazone, ≥ 97%, Sigma), Carbenecillin (Sigma), Aztreonam (Sigma), ethidium bromide (Invitrogen), and E. coli DH5α (Invitrogen) were purchased and used as received. P. aeruginosa strains (WT, NalB1, ΔMexB, ΔMexA,B-OprM) and plasmid (pMMB67EH) were provided by Hiroshi Yoneyama [131, 132]. All other reagents except indicated were purchased from Sigma and used as received. The nanopure deionized (DI) water (18 MΩ water, Barnstead) was used to prepare all solutions. The L-broth (LB) medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl in DI water, pH = 7.2) was prepared.

Design and construction of EGFP-mexB fusion gene

We fused N-terminal of mexB gene with C-terminal EGFP to prepare the EGFP-mexB fusion gene by asymmetrical PCR amplification, and then inserted it into the vector (pMMB67EH) to prepare pMMB67EH-EGFP-MexB vector [133]. We first used one pair of egfp primers (egfp primer-1: 5'-CTT GTC GAC AAG GGG ATC CAC CAT GGT GAG CAA GG-3' and egfp primer-2: 5'-CAA TGA AAA ACT TCG ACA TCT TGT ACA GCT CGT CCA TGC-3') and EGFP plasmid as a template to amply EGFP gene. We then used only egfp primer-1 and
amplified double stranded \textit{EGFP} gene as the template to create the coding strand of \textit{EGFP} gene (ss-egfp). Using the similar approaches, we used one pair of \textit{mexB} primers (\textit{mexB} primer-1: 5'-GCA TGG ACG AGC TGT ACA AGA TGT CGA AGT TTT TCA TTG-3', \textit{mexB} primer-2: 5'-GAT AAG CTT ATC ATT GCC CCT TTT CGAC-3') and genomic DNA of \textit{P. aeruginosa} as the template to generate and amplify the \textit{mexB} gene. We used \textit{mexB} primer-2 and the amplified double stranded \textit{mexB} genes as the template to generate and amply the template strand of \textit{mexB} gene (ss-\textit{mexB}), which has 20 bases that are complimentary to the ss-\textit{egfp} at the 3' ends. By mixing the ss-\textit{egfp} with ss-\textit{mexB}, it creates 20 bp double stranded DNA, that links the ss-\textit{egfp} with ss-\textit{mexB}, which is used as the template to generate and amply \textit{egfp-\textit{mexB}} fusion gene using both \textit{egfp} primer-1 and \textit{mexB} primer-2 as primers. The PCR products of the \textit{egfp-\textit{mexB}} fusion gene were characterized by agarose gel electrophoresis (0.8%, \textit{E} = 8-10 V/cm), and purified by the gel purification kit (Qiagen).

The purified PCR products of \textit{EGFP-\textit{mexB}} genes and vector (pMMB67EH) were digested by a pair of restriction enzymes (\textit{SalI/HindIII}), which was carried out by incubating the PCR products (10 ng) or the vector (5 ng) with two units of \textit{SalI/HindIII} each in the presence of 10x digestion buffer (4 or 3 μL) in DI water (40 or 30 μL) at 37 °C for 3 h, respectively. The digested products were characterized by the agarose gel electrophoresis and purified via the gel extraction kit. After purification, the digested and purified \textit{EGFP-\textit{mexB}} genes were ligated into the same treated vector (pMMB67EH) to produce pMMB67EH-
EGFP-MexB. The ligation was carried out by incubating digested and purified EGFP-mexB fusion genes (20 nM) with digested and purified vector (5 nM) in the presence of one unit of T4 DNA ligase and 10x ligation buffer (2 μL) in DI water (20 μL) at 12 °C overnight. All digestion, gel electrophoresis and ligation work are carried out using standard protocols [134].

The pMMB67EH-EGFP-MexB was then transformed into DH5α competent cells (Invitrogen). Briefly, we mixed the pMMB67EH-EGFP-MexB (10 μL, 0.1 μg/μL) with the competent cell suspension (100 μL, 2x10⁷ cells/mL), placed the mixture on ice for 30 min, followed by a heat shock at 42 °C for 90 s, then placed it back onto the ice for another 2 min. We then added the LB media (1 mL) to the mixture, incubated it in a shaker (LabLine Orbit-Environ, 37 °C, 200 rpm) for 1 h, and centrifuged the solution at 5000 rpm for 5 min. We removed the supernatant and suspended the pellets (transformed cells) in the fresh medium, spread them onto LB agar plates (0.5% yeast extract, 0.5% NaCl, 1% tryptone, 1.5% agar and 100 μg/ml ampicillin) and incubated the plates at 37 °C for 24 h. Positive ligations were verified by restriction enzyme digestion (SalI/HindIII), PCR amplification with use of primers (egfp primer-1 and mexB primer-2), and DNA sequencing (Applied Biosystem, 3730xl DNA Analyzer).

Plasmid with the correct sequence was finally transformed into MexB deleted strain (ΔMexB) using electroporation [135-138]. Briefly, the cells (ΔMexB) were cultured in the LB media under shaking at 37 °C until they reach the early log phase (OD₆₀₀ nm = 0.3-0.5). The cells were then harvested using
centrifugation (7000 g, 10 min, 4 °C), and washed with the sucrose (300 mM) three times, and resuspended in the sucrose. The cell suspension was placed on ice for 30 min before electroporation. We then mixed the competent ΔMexB cells (80 μL) with the fusion plasmid (pMMB67EH-EGFP-MexB, 10 μL, 1 μg/μL), transferred them to a pre-chilled sterile electroporation cuvette (gap between two electrodes = 0.2 cm), and applied the electric pulses (2.5 kV, 25 μF, 5 ms) (Biorad Gene Pulser). The cells were then cultured in the LB medium (3 mL) in the shaker (200 rpm, 37 °C) for 2 h, and harvested using centrifugation (5000 rpm, 5 min). The cells were streaked onto the LB agar plate containing 100 μg/ml carbenecillin, which was then incubated at 37 °C for 24 h. The clones were picked and cultured at 37 °C in the LB medium with 100 μg/mL carbenecillin to produce EGFP fused cells, MexA-EGFP-MexB-OprM.

Cell Culture and Assay

We pre-cultured each of five strains of \textit{P. aeruginosa}: WT, NalB1, ΔMexB, ΔMexA,B-OprM, and MexA-EGFP-MexB-OprM, by inoculating a single clone of each strain from the LB agar plates into the LB medium and placed them in the shaker (37 °C, 200 rpm) for 12 h. We then cultured the cells in the fresh LB medium in the shaker (37 °C, 200 rpm) for another 8 h, except adding 2 mM IPTG and 100 μg/mL carbenecillin in the medium for the culture of MexA-EGFP-MexB-OprM cells to induce the expression of the fusion protein as the OD$_{600\text{nm}}$ (optical density, absorbance, at 600 nm) of the cell suspension reaches 0.5. We harvested the cells using centrifugation (Beckman J2-21, JA-14 rotor, 6000 rpm,
23 °C, 10 min), washed them with the PBS buffer (50 mM phosphate, 100 mM NaCl, pH 7.0) three times, and resuspended them in the buffer. The cell concentration in the buffer (OD_{600 nm} = 0.1) was used to characterize the expression of the fusion protein (EGFP-MexB) in single live cells using fluorescence microscopy and to study the efflux function of membrane transporters by measuring accumulation kinetics of EtBr dyes in the cells using fluorescence spectroscopy.

Characterization of expression of fusion gene in single living cells using fluorescence microscopy

The cells suspended in the PBS buffer were imaged in a microchamber using dark-field optical microscopy and epi-fluorescence microscopy (Nikon, E-400) equipped with a CCD camera (Micromax, Roper Scientific) [71, 75, 90]. The design and construction of the microchamber and dark-field optical microscopy for imaging of single cells were fully described in our previous studies [71, 90]. In this study, the fluorescence filter cube (Chroma Tech) containing a band-pass excitation filter (455 ± 30 nm), band-pass emission filter (525 ± 30 nm) and a dichroic mirror (500 nm), was used for imaging expression of EGFP-MexB fusion protein in the strain (MexA-EGFP-MexB-OmrM). The dark-field optical microscope is equipped with a dark-field condenser (Oil 1.43-1.20, Nikon) and a 100x objective (Nikon Plan fluor 100x oil, iris, SL. N.A. 0.5-1.3, W.D. 0.20 mm).
Fluorescence spectroscopic study of transport of EtBr dye molecules in living cells

Fluorescence intensity of EtBr (10 and 40 μM) incubated with the cells (OD$_{600\text{ nm}}$ = 0.1) in the PBS buffer in the presence or absence of 0.1 mM CCCP were acquired over time (1-2 h) with 3-s time interval at room temperature using a fluorescence spectrometer (Perkin-Elmer, LS50B). The excitation and emission wavelengths were selected at 465 and 600 nm with a width of each slit at 10 nm, respectively.

Minimum inhibitory concentration (MIC) studies for different *Pseudomonas aeruginosa* strains

We measured the MICs of two representative antibiotics (AZT and CP) toward the cells: WT, NalB1, ΔMexB, MexA-(EGFP-MexB)-OprM, and ΔMexA,B-OprM. Each strain was pre-cultured by inoculating a single clone from the LB agar plates into the LB medium (add 100 μg/mL carbenecillin for culturing the EGFP fusion strain to maintain the plasmid) and shaken overnight (200 rpm, 37 °C). The cell suspension (100 μL, ~10$^4$ cells) was added into the fresh LB medium (2.9 mL) in the presence of a set of different concentrations of AZT or CP (add 100 μg/mL carbenecillin and 2 mM IPTG in the culture of the fusion strain to induce the expression of fusion protein), and shaken for another 16 h (200 rpm, 37 °C). The concentration and number of the cells for each cultured strain was measured using the UV-visible spectroscopy (OD$_{600\text{ nm}}$) and dark-field microscopy, respectively.
CHAPTER V

SIZE-DEPENDENT ANTIBACTERIAL EFFECTS OF SILVER NANOPARTICLES CONJUGATED ANTIBIOTICS ON GRAM-NEGATIVE BACTERIA, *PSEUDOMONAS AERUGINOSA*

INTRODUCTION

The development of nanotechnology presents us new therapeutic and diagnostic concepts in a wide variety of biological and medical processes. Due to their unique characteristics, nanoparticles are considered to have wide applications in biosensing [139], bioimaging [140] and targeted drug delivery [141]. For example, nanoparticles are used to enhance oral delivery by improving the solubility of poorly absorbed drugs [142]. They are capable of penetrating the cells to help cellular internalization and connective tissue permeation, thus delivering the drugs efficiently to the targeted tissue without clogging capillaries [143, 144]. The basic requirements for targeted drug delivery are that the carrier should be circulated extensively in the bloodstream, it must be small enough to access target tissues and target cells, it has to be capable of delivering the loaded cargos into the cells, and following endocytosis, it must be able to escape endosome–lysosome processing. Different types of nanoparticles with biomedical relevance such as polymeric nanoparticles, dendrimers, liposomes, quantum dots, metal nanoparticles, etc. have been extensively studied in the past [145]. Of them, noble particles like Ag or Au NPs have been used to delivery protein-based drugs and are of particular utility because the unique
optical, chemical and electronic properties of noble NPs made them well suited for biomedical sensing, therapeutic drug delivery [26, 44, 71, 90, 97, 145].

With significant developments in the understanding of nanosystems, research efforts are focused on integrating them with biology. Resistance of bacteria to bactericides and antibiotics has been a research subject of great interest due to the development of multidrug resistant strains [2, 9, 12, 17]. The resistance to bactericides or antibiotics is largely because of active efflux of antibiotics outside cells by multidrug resistance (MDR) transporters on the membranes [1-12, 45, 46, 56, 62, 146]. Numerous researchers have explored the feasibility and efficiency of using nanomaterials as new drug carriers. For instance, Gu et al. reported the enhanced antimicrobial effects of nanoparticles conjugated with vancomycin [147]. Grace et al. used the gold nanoparticles conjugated with drugs and investigated their antimicrobial effects in various types of organisms [148]. Other researches also characterized the enhanced antibacterial effects of drug conjugated silver nanoparticles [149-151]. All these research results demonstrated enhanced antibacterial effects of nanoparticles when conjugated with different drugs, which raises hopes in applying nanoparticles as effective drug carriers for the specific targeting and treatment of many MDR related diseases. While there were many reports about the delivery of different drugs in nanoparticle conjugated forms, little efforts were made, so far, to determine how the sizes of nanoparticle carriers would impact on the efficiency of antibiotic therapies.
Hence, in this study, we compared the therapeutic efficiency and biocompatibility of AgMUNH₂-OFLX NPs with respect to their carrier sizes (2.4 ± 0.7 nm, 13.0 ± 3.1 nm and 92.6 ± 4.4 nm) and drug free forms (OFLX). Notably, we found the size-dependent antimicrobial effects of silver NPs conjugated antibiotics (OFLX) on gram-negative bacteria, *P. aeruginosa*, which were that larger sized NPs conjugated antibiotics generated higher inhibitory effects against the growth of bacteria than smaller sized NPs conjugated antibiotics at the same concentration of conjugated antibiotics and two of larger sized NPs conjugated antibiotics showed lower MIC values than their drug free forms. Thus our results offer new insights into design of more effective drug delivery vehicles and suggest that antibiotics conjugated with silver NPs might be used in therapy for better treatment of multidrug resistance associated diseases.

RESULTS AND DISCUSSION

Synthesis and characterization of different sized silver (Ag) nanoparticles

First, we have synthesized three sized Ag NPs (2.4 ± 0.7 nm, 13.0 ± 3.1 nm and 92.6 ± 4.4 nm) according to the methods described in the experimental section. Next, we characterized these three sized Ag NPs using UV-visible spectroscopy (Fig. 25A) and the results demonstrated that 2.4 ± 0.7 nm silver NPs showed the peak of wavelength at 392 nm (Fig. 25A, I), while 13.0 ± 3.1 and 92.6 ± 4.4 nm Ag NPs showed the peak wavelengths at 398 nm (Fig. 25A, II) and 482 nm (Fig. 25A, III), respectively. We also characterized their sizes using high resolution transmission electron microscopy (HRTEM) (Fig. 25B) and the
corresponding histograms of size distribution of each sized Ag NPs (Fig. 25C) showed that the average diameters of three sized Ag NPs are $2.4 \pm 0.7$, $13.0 \pm 3.1$ and $92.6 \pm 4.4$ nm, respectively. Taken together, those results are quite consistent with our previous studies [38, 42, 43].

**Conjugation of ofloxacin (OFLX) to Ag NPs and characterization of Ag carriers by UV-visible spectroscopy**

After synthesis of three sized Ag NPs, they were coated with MUNH$_2$ groups first before linking with the drug molecules (OFLX). After conjugation, OFLX conjugated NPs solutions were centrifuged and washed extensively to remove any unbound drug molecules. Successful conjugation was verified by the red shift in the UV-visible spectra before and after conjugation. As shown in Fig. 26A-C, the UV-visible spectra of each sized Ag, AgMUNH$_2$, AgMUNH$_2$-OFLX NPs all showed a red-shift of peak wavelength as Ag NPs (Fig. 26A-C, a) are conjugated with MUNH$_2$ groups first (Fig. 26A-C, b). Then, there were another red-shift of peak wavelength and the emergence of the peak wavelength of OFLX at around 325 nm as each sized AgMUNH$_2$ NPs were finally linked with OFLX molecules (Fig. 26A-C, c). All these results suggest that each sized Ag NPs are successfully conjugated with MUNH$_2$ groups and OFLX drug molecules step by step. [For abbreviation, Ag, AgMUNH$_2$, AgMUNH$_2$-OFLX NPs of three sizes will be named as 2.4 nm Ag carriers (A), 13.0 nm Ag carriers (B) and 92.6 nm Ag carriers (C), respectively].
Fig. 25 Synthesis and characterization of different sized silver nanoparticles.

(A) UV-visible spectra of three sized Ag NPs. (B) Representative TEM images of three sized Ag NPs. Scale bars are shown in each image. (C) Histograms of size distribution of three sized Ag NPs from TEM images. (I) 2.4 ± 0.7 nm Ag NPs, (II) 13.0 ± 3.1 nm Ag NPs and (III) 92.6 ± 4.4 nm Ag NPs
Fig. 26 Characterization of three sized Ag, AgMUNH$_2$ and AgMUNH$_2$-OFLX NPs by UV-visible spectroscopy.

UV-visible spectra of (A) 2.4 nm Ag carriers, (B) 13.0 nm Ag carriers, (C) 92.6 nm Ag carriers; a- Ag NPs, b- AgMUNH$_2$ NPs, c- AgMUNH$_2$-OFLX NPs
Characterization of Ag, AgMUNH₂ and AgMUNH₂-OFLX NPs by DFOMS and LSPR Spectroscopy

Aside from the characterization of Ag, AgMUNH₂, AgMUNH₂-OFLX NPs by UV-visible spectroscopy as shown above, we also characterized them using our Dark Field Optical Microscopy Spectroscopy (DFOMS) system [37, 38, 42-44, 98]. In addition, we measured the representative LSPR spectra of each sized Ag, AgMUNH₂ and AgMUNH₂-OFLX NPs using our Nuance multispectral imaging system (CRI).

Fig. 27 showed the representative dark field images and LSPR spectra of each sized Ag, Ag MUNH₂ and AgMUNH₂-OFLX NPs. As shown in Fig. 26, for each sized Ag carriers, the peak wavelengths of Ag carriers start to shift into red direction as Ag NPs are conjugated with MUNH₂ groups and followed by another red shift when AgMUNH₂ groups are linked with OFLX drug molecules. In consistence with the above UV-visible spectra results, we also found that the peak wavelengths of representative LSPR spectra of each sized Ag, AgMUNH₂, AgMUNH₂-OFLX NPs showed a stepwise red shift from Ag NPs to AgMUNH₂ NPs, and to AgMUNH₂-OFLX NPs as depicted in Fig. 27, which showed representative dark-field images and LSPR spectra of 2.4 nm (A), 13.0 nm (B) and 92.6 nm (C) Ag carriers, respectively. The dark-field images of each type of Ag carriers are in the order of Ag (a), AgMUNH₂ (b) and AgMUNH₂-OFLX (c) NPs and the corresponding LSPR spectra of Ag carriers (d, i-vi) are shown as labeled in Fig. 27A-C, a (i-ii), b (iii-iv) and c (v-vi).
For instance, 2.4 ± 0.7 nm Ag NPs in Fig. 27A-a are mostly blue and they will appear greenish when they are conjugated with MUNH₂ groups (Fig. 27A-b) and even more greenish in AgMUNH₂-OFLX NPs (Fig. 27A-c). Besides, the peak wavelengths of representative LSPR spectra of 2.4 nm Ag carriers also showed a red shift from Ag NPs to AgMUNH₂ NPs, and to AgMUNH₂-OFLX NPs (i-vi of Fig. 27A-d). The other two sized Ag carriers have also shown a stepwise red shift in the peak wavelengths of representative LSPR spectra from bare Ag NPs to AgMUNH₂ NPs, and to AgMUNH₂-OFLX NPs as shown in Fig. 27B and C (Fig. 27B- 13.0 nm Ag carriers, Fig. 27C- 92.6 nm Ag carriers).

Those above results demonstrated that each sized Ag NPs were successfully synthesized and conjugated with MUNH₂ groups and OFLX molecules step by step, and their optical properties were also well characterized by UV-visible spectroscopy, DFOMS and LSPR spectroscopy.

**Calculation of conjugation ratios of OFLX molecules to AgMUNH₂ NPs in each sized AgMUNH₂-OFLX NPs**

Briefly, the standard UV-visible spectra curves of OFLX alone at different concentrations were obtained first to calculate the extinction coefficient of OFLX solution. Then, the concentration of conjugated OFLX molecules in each sized AgMUNH₂-OFLX NPs was calculated from the net spectra of conjugated drugs by subtracting the UV-visible spectra of AgMUNH₂-OFLX and AgMUNH₂ nanoparticle solution of the same concentrations. After that, the conjugation
Fig. 27 Characterization of three sized Ag, AgMUNH$_2$ and AgMUNH$_2$-OFLX NPs by DFOMS and LSPR spectroscopy.

Representative dark-field images and LSPR spectra of 2.4 nm (A), 13.0 nm (B) and 92.6 nm (C) Ag carriers, respectively. Dark-field images of bare Ag NPs (a), AgMUNH$_2$ NPs (b) and AgMUNH$_2$-OFLX (c) NPs and representative LSPR spectra (d) of single Ag, AgMUNH$_2$, AgMUNH$_2$-OFLX NPs (i-ii: Ag NPs, iii-iv: AgMUNH$_2$ NPs, v-vi: AgMUNH$_2$-OFLX NPs )
ratios of OFLX molecules to AgMUNH₂ NPs for each sized AgMUNH₂-OFLX NPs (Table 4) are calculated from dividing the concentration of conjugated OFLX molecules by the concentration of that sized AgMUNH₂-OFLX NPs solution.

As shown in Table 4, the conjugation ratios of OFLX molecules to single AgMUNH₂ NP in AgMUNH₂-OFLX NPs are 863:1, 9435:1 and 652941:1 for the 2.4 nm, 13.0 nm and 92.6 nm sized AgMUNH₂-OFLX NPs, respectively.

Table 4 Calculation of conjugation ratios of ofloxacin (OFLX) molecules to each AgMUNH₂ nanoparticle in each sized AgMUNH₂-OFLX NPs

<table>
<thead>
<tr>
<th>AgMUNH₂-OFLX Diameter (nm)</th>
<th>AgMUNH₂-OFLX concentration (nM)</th>
<th>Net absorbance of OFLX at 288 nm</th>
<th>Concentration of conjugated OFLX (µM)</th>
<th>Conjugation ratio of OFLX to AgMUNH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>50</td>
<td>0.337</td>
<td>43.152</td>
<td>863.0</td>
</tr>
<tr>
<td>13.0</td>
<td>3.3</td>
<td>0.243</td>
<td>31.136</td>
<td>9435.0</td>
</tr>
<tr>
<td>92.6</td>
<td>0.02975</td>
<td>0.152</td>
<td>19.425</td>
<td>652941.8</td>
</tr>
</tbody>
</table>

a \( \lambda_{\text{max}} = 288 \text{ nm} \)

b \( \epsilon (1/\text{uM cm}), 288 \text{ nm} = 0.0078 \)

Stability study of AgMUNH₂-OFLX NPs in modified LB medium

Stability studies of each sized AgMUNH₂-OFLX NPs in the culture medium are very crucial prerequisites for further assessment of the antibacterial efficiency of these NPs. Preliminary studies indicated all three sized AgMUNH₂-NPs are
not stable in the LB medium (data not shown), so we modified the LB medium. To study the stability of NPs in the modified medium, we determined the number of single NPs using DFOMS as described previously [38, 39]. The concentrations of each sized AgMUNH$_2$-OFLX NPs used here were 6 nM, 0.08 nM and 7 pM for 2.4, 13.0 and 92.6 nm AgMUNH$_2$-OFLX NPs, respectively (Note that for each sized NPs, the concentration used to study their stabilities in the culture medium here was much higher than that required for the later MIC experiments). A minimal of 20 images at each time point from representative locations for each experiment was acquired and the experiment was repeated three times. Results were shown in Fig. 28A-C, which represented the average number of AgMUNH$_2$-OFLX NPs per image versus time in modified LB medium for 2.4 nm, 13.0 nm and 92.6 nm sized AgMUNH$_2$-OFLX NPs, respectively.

Notably, for each sized AgMUNH$_2$-OFLX NPs (2.4 nm, 13.0 nm and 92.6 nm, respectively), the average number of NPs per image versus time remained unchanged over 24 h of incubation (Fig. 28A-C). This indicated that all sized particles were stable in the modified LB medium for at least 24 hrs at the studied concentrations. These positive results paved the way for further utilization of these silver nanoparticles conjugated antibiotics in gram-negative *Pseudomonas aeruginosa* cells in order to assess their potential antibacterial effects by measuring the MIC values of silver nanoparticles conjugated OFLX compared to their free forms in different *P. aeruginosa* strains containing different level of expression of the MexA,B-OprM pump proteins (Wide type - PAO1 and MexA,B-OprM efflux pump deleted strain - ΔMexA,B-OprM).
Fig. 28 Stability study of three sized AgMUNH₂-OFLX NPs in modified LB medium for over 24 hrs by DFOMS.

Results are shown as the average number of particles per image taken from a high resolution color camera for each sample prepared in a micro-channel slide. Concentrations of three sized AgMUNH₂-OFLX NPs used in this study are 6nM, 0.8 nM and 7 pM for 2.4 nm (A), 13.0 nm (B) and 92.6 nm (C) sized AgMUNH₂-OFLX NPs, respectively.
Besides, we also studied the stability of those AgMUNH$_2$-OFLX NPs in modified LB medium by UV-visible spectroscopy \[38, 39, 97, 98\]. As shown in Fig. 29, the UV-visible spectra of each sized AgMUNH$_2$-OFLX solution remain the same over 24 h of incubation. Combining the results of stability studies by DFOMS above and UV-visible spectroscopy, all indicated that three sized AgMUNH$_2$-OFLX NPs are stable in the modified LB medium over the 24 hrs of time.

**Cells function properly in the modified LB medium**

After we verified that three sized AgMUNH$_2$-OFLX NPs are stable in the modified LB medium for at least 24 hrs, our next step would be to check the efflux functions of different *P. aeruginosa* strains in the modified LB medium. We used the time course incorporation of fluorescent dyes (EtBr, 10 \(\mu\)M) to probe the efflux functions of different *P. aeruginosa* strains as previously studied \[38, 99\]. As shown in Fig. 30, for each of strains, efflux kinetics of EtBr molecules by each of three *P. aeruginosa* strains were nearly the same in both LB medium (A) and modified LB medium (B), with the highest accumulation of intracellular EtBr molecules in the pump deleted strain (AMexA,B-OprM), followed by a moderate accumulation of EtBr in wide type strain (PAO1) and the lowest accumulation of EtBr in the pump overexpressed strain (NalB). This indicated that modified LB medium was fully capable of maintaining original efflux functions of these strains. Those results are quite consistent with previous studies from our and other groups \[38, 71, 73, 99\].
Fig. 29 Stability study of three sized AgMUNH$_2$-OFLX NPs in modified LB medium by UV-visible spectroscopy.

The UV-visible spectra of each sized AgMUNH$_2$-OFLX solution in modified LB medium at t 0h (a) and t 24h (b) are shown as 2.4 nm AgMUNH$_2$-OFLX (A), 13.0 nm AgMUNH$_2$-OFLX (B) and 92.6 nm AgMUNH$_2$-OFLX (C), respectively.
Fig. 30 Time course incorporation of EtBr (10 μM) in different strains of *Pseudomonas aeruginosa* cultured in LB and modified LB medium.

Different *P. aeruginosa* strains were cultured in LB (A) and in modified LB medium (B), respectively. (a) Pump deleted ΔMexA,B-OprM strain, (b) Wide type PAO1 strain, (c) Pump overexpressed NalB strain
Fig. 31 Characterization of the viability of single *P. aeruginosa* cells using live/dead bac/Light viability assay.

(A) Dark field optical image and (B) green fluorescence images of WT PAO1 cells cultured in LB medium in the cell viability assay, respectively. (C) Plots of percentage of viable cells (%) in LB (a) and modified LB medium (b) for each of three *P. aeruginosa* strains, respectively.
Next, we did the cell live / dead viability assay at the end of cell function studies to see if the number of viable or dead cells grew in these two mediums is approximately the same as previously studied [37, 38]. Fig. 31A-B showed the representative dark-field and fluorescence images of cells in this viability assay, more than five hundred cells from multiple images are used to calculate the number of viable cells for each strain in either regular or modified LB medium. As shown in Fig. 31C, for each of three *P. aeruginosa* strains, the number of viable cells in two types of LB mediums is nearly the same (about 98%).

**Size dependent antimicrobial effects of AgMUNH\(_2\)-OFLX NPs in different *P. aeruginosa* strains**

To investigate the antimicrobial effects of AgMUNH\(_2\)-OFLX NPs in *P. aeruginosa* cells, MICs of AgMUNH\(_2\) conjugated OFLX were determined for each sized NPs with the respective free forms of OFLX and corresponding AgMUNH\(_2\) NPs as controls. At the end of the experiment, bulk images of glass tubes for each group of nano-carriers were taken as shown in Fig. 32-34 for PA01 and ΔMexA,B-OprM strains, respectively. Those images clearly demonstrated that 92.6 nm sized AgMUNH\(_2\) NPs conjugated OFLX molecules generated the highest antibacterial effects followed by 13.0 nm and 2.4 nm sized AgMUNH\(_2\) NPs conjugated OFLX at the same concentration of conjugated OFLX molecules, no matter which strain was used.

Optical densities at 600 nm of samples in each glass tube were measured and results were plotted against the concentrations of conjugated OFLX
molecules as shown in Fig. 34. The MIC curves showed the same trend of antimicrobial effects generated by different sized AgMUNH$_2$-OFLX NPs as we observed from the glass tube images in Fig. 32-34, which is that larger sized AgMUNH$_2$ NPs conjugated OFLX molecules generated more inhibitory effects on the growth of bacteria in both strains. Those results all implied the size-dependent inhibitory effects of AgMUNH$_2$ NPs conjugated OFLX molecules on the growth of gram-negative bacteria, *P. aeruginosa*.

We also found that the drug deficient AgMUNH$_2$ groups did little impact on the cell survivals compared to their counterparts with drug conjugated at the same concentrations, which implied that AgMUNH$_2$ NPs posed little toxic or antimicrobial effects when Ag NPs were coated with extra MUNH$_2$ groups on their surfaces, though several reports have shown the antibacterial effects of pure silver NPs [150, 152]. In addition, our results indicated that toxic effects of silver NPs could be effectively circumvented when other chemical groups are coated on their surfaces, which is very important for future application of silver NPs as effective drug carriers for specifically targeting and treating diseased areas without harming the surrounding health cells.

Besides, we can also see that as the size of silver NPs increases, it also leads to higher antimicrobial effects since MIC values of conjugated OFLX in PAO1 cells decreased from > 2.16 uM (2.4 nm AgMUNH$_2$-OFLX NPs) to 1.08 uM (13.0 nm AgMUNH$_2$-OFLX NPs), and to 0.6 nM (92.6 nm AgMUNH$_2$-OFLX NPs) (Table 5, Fig. 34A).
Fig. 32 Images of glass tubes showing MIC study of OFLX in PAO1 cells.

(A) OFLX alone group, tubes (a-i) represent different concentrations of OFLX in each glass tube as 0, 0.2, 0.4, 0.6, 0.8, 1.08, 1.62, 2.16 μM, respectively. (B) 2.4 nm AgMUNH$_2$-OFLX NPs, the conjugated concentrations of OFLX in tubes (a-h) are 0.2, 0.4, 0.6, 0.8, 1.08, 1.62, 2.16 μM, respectively; tube (i) contains 2.16 μM of 2.4 nm AgMUNH$_2$ control NPs. (C) 13.0 nm AgMUNH$_2$-OFLX NPs. (D) 92.6 nm AgMUNH$_2$-OFLX NPs, the concentrations of OFLX in tubes (Fig. 32C-D, a-i) are identical to the ones in Fig. 32B, tube (i) also contained the AgMUNH$_2$ control NPs of 13.0 and 92.6 nm, respectively. For all sized AgMUNH$_2$-OFLX NPs, the concentration of conjugated OFLX is calculated based on the concentration of AgMUNH$_2$-OFLX NPs solution and the conjugation ratio of OFLX to that sized AgMUNH$_2$ NPs.
Fig. 33 Images of glass tubes showing MIC study of OFLX in ΔMexA,B-OprM cells.

(A) OFLX alone group, glass tubes (a-i) represent different concentrations of OFLX in each tube as 0, 0.02, 0.04, 0.06, 0.08, 0.11, 0.14, 0.27 μM, respectively.

(B) 2.4 nm AgMUNH₂-OFLX NPs, the conjugated concentrations of OFLX in tubes (a-h) are 0.02, 0.04, 0.06, 0.08, 0.11, 0.14, 0.27 μM, respectively; tube (i) contains 0.27 μM of 2.4 nm AgMUNH₂ control NPs. (C) 13.0 nm AgMUNH₂-OFLX NPs. (D) 92.6 nm AgMUNH₂-OFLX NPs, the concentrations of OFLX in tubes (Fig. 33C-D, a-i) are identical to the ones in Fig. 33B, tube (i) also contained the AgMUNH₂ control NPs of 13.0 and 92.6 nm, respectively. The concentrations of conjugated OFLX for all sized AgMUNH₂-OFLX NPs are calculated using the same approaches as described in Fig. 32.
Fig. 34 MICs of OFLX in PAO1 and ΔMεxA, B-OprM cells.

Optical density values (OD_{600nm}) of each sample in wt PAO1 (A) and ΔMεxA, B-OprM (B) cells are plotted against the concentrations of conjugated OFLX (μM) at the end of MIC experiment; (a) 2.4 nm AgMUNH₂, (b) 13.0 nm AgMUNH₂, (c) 92.6 nm AgMUNH₂, (d) 2.4 nm AgMUNH₂-OFLX, (e) OFLX alone, (f) 13.0 nm AgMUNH₂-OFLX and (g) 92.6 nm AgMUNH₂-OFLX. Error bars represent the standard deviations from three repeated experiments for each sample.
Table 5 MICs of AgMUNH₂ NPs conjugated OFLX in PAO1 and ΔMexA,B-OprM cells

<table>
<thead>
<tr>
<th>AgMUNH₂-OFLX types</th>
<th>MICs of conjugated OFLX in AgMUNH₂-OFLX NPs (μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAO1</td>
<td>ΔMexA,B-OprM</td>
</tr>
<tr>
<td>2.4 nm</td>
<td>&gt; 2.16</td>
<td>&gt; 0.27</td>
</tr>
<tr>
<td>13.0 nm</td>
<td>1.08</td>
<td>0.14</td>
</tr>
<tr>
<td>92.6 nm</td>
<td>0.6</td>
<td>0.06</td>
</tr>
<tr>
<td>OFLX alone</td>
<td>2.16</td>
<td>0.27</td>
</tr>
</tbody>
</table>

The results from MIC studies in the ΔMexA,B-OprM strain (Table 5, Fig. 34B) showed the similar trend as seen in wide type PAO1 strain. First, drug free AgMUNH₂ NPs of three sizes had little or no antibacterial effects. Secondly, drug conjugated AgMUNH₂-OFLX NPs exhibited antibacterial effects as a function of size of AgMUNH₂ NPs.

For example, as the size of AgMUNH₂ NPs increased from 2.4 nm to 13.0 nm, and to 92.6 nm, the MICs of OFLX for these different sized AgMUNH₂-OFLX NPs in ΔMexA,B-OprM strain were decreased from > 0.27 μM to 0.14 μM and to 0.06 μM, respectively. From these results, we found that 2.4 nm AgMUNH₂-OFLX NPs are probably the most biocompatible NPs since their MIC values are close to those in the OFLX drug alone group for both strains while the other two sized AgMUNH₂-OFLX (13.0 nm and 92.6 nm) can generate more antimicrobial effects when they are conjugated with drugs.
Those interesting results not only confirm the existence of enhanced antimicrobial effects of the drug (OFLX) conjugated silver NPs but also suggest those effects are largely dependent on the size of NP carriers. As the size of particles increases, their surface areas also tremendously enlarge which made it possible for conjugating larger number of drug molecules to single particle. These particles with higher conjugation ratios of drug molecules will offer greater advantages over those with lower conjugation ratios for targeting and binding with specific biological molecules and conferring the potent effects of loaded drugs. These phenomena have been observed in many previous studies [33, 35, 40, 147, 149]. For example, Burygin et al. found enhanced antibacterial activity of antibiotics mixed with gold NPs [40] and Bhattacharya et al. found that gold NPs conjugated drugs showed greater bactericidal activity and reduced MIC compared to their respective free forms [35]. Though these studies observed the enhanced antimicrobial effects from drug or antibiotics conjugated NPs, it remains unanswered about the exact mechanisms governing these enhanced antibacterial effects.

SUMMARY

In this study, we have successfully synthesized three sized silver NPs (2.4 ± 0.7, 13.0 ± 3.1 and 92.6 ± 4.4 nm) and conjugated them with antibiotic (OFLX). We also modified the regular LB medium and found that different P. aeruginosa strains cultured in modified LB medium can still maintain their original efflux functions as compared with those cultured in regular LB medium without
significant changes in terms of cell function and cell viability. Next, we found size-dependent antimicrobial effects generated by these different sized AgMUNH2-OFLX NPs in one of gram-negative, clinically important bacteria, *P. aeruginosa*, with larger sized AgMUNH2-OFLX (92.6 nm) NPS showing the highest bactericidal effects, followed by a middle sized AgMUNH2-OFLX (13.0 nm) and finally the smallest sized AgMUNH2-OFLX (2.4 nm) which result is quite similar to that of the antibiotic alone group. These results indicate that 2.4 nm silver NPs are probably the most biocompatible silver NPs for sensing and imaging bio-molecules while the other bigger sized silver NPs, due to their higher efficiency of antibacterial effects, can be used as efficient drug carriers in the treatment of many diseases.

In summary, our studies clearly showed the existence of size-dependent antibacterial effects of AgMUNH2 NPs conjugated OFLX and these important findings will aid in the application of nano-materials conjugated with different types of drugs for cancer therapy in the future.

**METHODS**

**Reagents and supplies**

Silver perchlorate monohydrate (99%, Alfa Aesar), sodium citrate dihydrate (99%, Sigma), 2-mercaptoethanol (99%, Sigma), 11-amino-1-undecanethiol hydrochloride (MUNH2, 99%, Sigma) and sodium borohydride (98%, Sigma) were used, as received, for synthesis of Ag NPs. Live/dead bacLight viability and counting assay were purchased from Invitrogen; all other
reagents like LB powder, ofloxacin (OFLX), were purchased from Sigma-Aldrich. 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC, 99%) and N-hydroxysulfosuccinimide (s-NHS, 98.5%) were from Pierce.

Synthesis and characterization of different sized silver (Ag) nanoparticles

Three different sized Ag NPs (2.4 ± 0.7 nm, 13.0 ± 3.1 nm and 92.6 ± 4.4 nm) were synthesized and purified as described previously [38, 42, 43]. Briefly, for the synthesis of 2.4 ± 0.7 nm Ag NPs, 0.11 mM of AgNO₃, 1.91 mM of sodium citrate, 0.052 mM of PVP, and 25.0 mM of H₂O₂ in nanopure water (42.3 mL) were prepared freshly, mixed and stirred constantly. Then, we added NaBH₄ (150 µL, 100 mM) into the mixture, the solution color turned to light yellow. After stirring for another 3 h, the solution was then filtered using 0.2 µm membrane filters (Whatman). For the synthesis of 13.0 ± 3.1 nm Ag NPs, AgClO₄ (2.5 mL, 10 mM, ice-cold) was added into the stirring mixture (247.5 mL) of ice-cold sodium citrate (3 mM) and NaBH₄ (10 mM), and the mixture was stirred at room temperature for additional 4 h. The solution was then filtered using 0.22 µm filters (Whatman) and washed twice with nanopure DI water using ultracentrifugation at 15000 RCF (relative centrifugal force), to prepare stable and purified Ag NPs. For the synthesis of 92.6 ± 4.4 nm Ag NPs, we added sodium citrate (34 mM, 10mL) into a refluxing (100 °C) stirring aqueous solution of AgNO₃ (3.98 mM, 500 mL) and stirred it for 35 min. Then, we stopped stirring and heating the mixture and allowed it to cool to room temperature. We added additional sodium citrate (2.5 mM) to further stabilize the NPs in solution. The solution was then filtered via
0.22 μm filters and washed three times using nanopure DI water via centrifugation. The purified Ag NPs were dispersed in the PBS buffer (0.5 mM phosphate buffer, 1.5 mM NaCl, pH=7.0). The sizes of single NPs were determined using high-resolution transmission electron microscopy (HRTEM) (FEI Tecnai G2 F30 FEG). The concentrations of NPs were measured and calculated, as described previously [38, 42, 43]. Plasmonic absorption and scattering properties of bulk NPs were characterized using UV–visible spectrometry (Hitachi).

Conjugation of ofloxacin (OFLX) to AgMUNH₂ NPs and calculation of conjugation ratios of drug molecules to AgMUNH₂ NPs

1 mL 100 mM MUNH₂ in ethanol was added to 100 mL freshly prepared Ag NPs of three different sizes (2.4 ± 0.7, 13.0 ± 3.1 and 92.6 ± 4.4 nm), respectively. The mixtures were stirred for 24 h to attach MUNH₂ onto the surface of NPs via their interaction of thiol groups with NPs. The AgMUNH₂ NPs were washed twice by nanopure water to remove excess MUNH₂ using centrifugation (Beckman Optima L90k), 30k rpm (60 min) for 2.4 ± 0.7 nm AgMUNH₂, 12k rpm (90 min) for 13.0 ± 3.1 nm AgMUNH₂ and 2.5k rpm (20 min) for 92.6 ± 4.4 nm AgMUNH₂ at 4 °C. The AgMUNH₂ NPs were immediately characterized using UV-visible spectroscopy, dark-field optical microscopy and spectroscopy (DFOMS), and dynamic light scattering (DLS). The amine groups of AgMUNH₂ NPs were conjugated to the carboxyl group of OFLX via peptide bonds using EDC and s-NHS as mediators. OFLX was first dissolved in 0.5 M HCl and then
diluted to 10 mM by MES buffer (pH 5.0, 50 mM). EDC (30 \mu mol) and s-NHS (150 \mu mol) were added to OFLX (3 mL, 50 mM), forming OFLX-s-NHS esters. After stirring at room temperature for 40 min, the reaction was stopped by adding 10 \mu l 2-mercaptoethanol. Then, 1 mL OFLX-s-NHS esters was added to 100 mL Ag MUNH$_2$ NPs of three different sizes, respectively. The solutions were mixed using a rotary shaker at room temperature for 3 h. The final products (AgMUNH$_2$-OFLX NPs of three different sizes) were washed using centrifugation, 30k rpm (60 min) for 2.4 nm AgMUNH$_2$-OFLX, 12k rpm (90 min) for 13.0 nm Ag MUNH$_2$-OFLX and 2.5k rpm (20 min) for 92.6 nm Ag MUNH$_2$-OFLX. The purified AgMUNH$_2$-OFLX NPs were resuspended in 50 mM PBS (pH 7.0) and stored at 4 °C for the future use. The sizes and optical properties of AgMUNH$_2$-OFLX NPs were characterized using DLS, UV-visible spectra and DFOMS.

Conjugation ratios of drug (OFLX) to each of three sized AgMUNH$_2$ NPs were calculated as described below. First, a standard curve was generated by measuring the absorbance value (A) at peak wavelength (\(\lambda = 288\) nm) from the absorption UV-visible spectra for different concentrations of OFLX alone solutions to calculate the absorption coefficient (\(\varepsilon\)). Next, to get the absorbance scale for each sized AgMUNH$_2$-OFLX NPs, we divided the absorbance value at \(\lambda = 288\) nm by corresponding height (mm) from the UV-visible spectra of each sized AgMUNH$_2$-OFLX solution at stock concentration (50 nM for 2.4 nm, 3.3 nM for 13.0 nm and 0.02975 nM for 92.6 nm AgMUNH$_2$-OFLX NPs, respectively). After that, we calculated the net height (cm) of absorbance at 288 nm corresponding to the part of conjugated OFLX by subtracting the height \(\lambda=288\) nm.
from spectra of AgMUNH$_2$-OFLX solution to the height $\lambda=288$ nm from spectra of AgMUNH$_2$ solution alone. Then, we got the concentrations of conjugated OFLX for each sized AgMUNH$_2$-OFLX NPs from each value of absorbance scale timing net height of absorbance for conjugated OFLX part. Finally the conjugation ratio for each sized AgMUNH$_2$-OFLX NPs is calculated by dividing the concentration of conjugated OFLX to its stock concentration of AgMUNH$_2$-OFLX solution, respectively.

**Stability study of AgMUNH$_2$-OFLX NPs in modified LB medium**

Studies of stability of AgMUNH$_2$-OFLX NPs in modified LB medium were done by monitoring the UV-visible spectra changes and directly countering the average number of NPs from 10 images by DFOMS on a slide prepared from the solution containing each sized AgMUNH$_2$-OFLX NPs in modified LB medium at four different time points over 24 h of incubation. The concentration used for each sized AgMUNH$_2$-OFLX NPs in those stability studies is much higher than required for the later MIC experiment according to previous MIC studies of OFLX alone in different *P. aeruginosa* strains [13]. All experiments have been repeated at least three times.

**Cell function and viability assay in modified LB medium**

To investigate if the cell functions have changed or not in the modified LB medium, we studied the efflux functions of different *P. aeruginosa* strains (wide type PAO1, NalB with efflux pump overexpressed and ΔMexA,B-OprM with
MexA,B-OprM pump deleted). A single colony from freshly streaked plates for each of three strains were inoculated into 15 mL regular and modified LB medium and cultured with vigorous shaking (200 rpm, 37 °C). Samples were taken out for measuring the optical density (OD600 nm) every 30 min up to 12 hrs of culture. Time course incorporation of ethidium bromide (EtBr, 10 µM) in the above P. aeruginosa strains were monitored over a two-hour period on a fluorescence spectrometer. The excitation and emission wavelength were set at 465 and 600 nm with a slit of 10 and 10 nm width, respectively.

Cell viability assay were done according to the manufacture’s instruction (Bac Live / Dead viability assay from Invitrogen). The percentage of live and dead cells were calculated from multiple images of both Dark Field and the corresponding Green / Red fluorescence images for over 500 cells in total for each strain used.

**Study of MIC of OFLX in different P. aeruginosa strains**

To investigate the antibacterial effects of silver NPs with or without drug molecule (OFLX) conjugated, we directly measured the MICs of OFLX in different P. aeruginosa strains for those different sized AgMUNH₂-OFLX NPs. For either PAO1 or ΔMexA,B-OprM strain used here, about 10⁴ cells from a pre-culture solution were inoculated into the modified LB medium containing a set of different concentrations of OFLX (concentrations of corresponding OFLX for three sized AgMUNH₂-OFLX NPs are calculated based on their conjugation ratios, respectively; AgMUNH₂ NPs of each size and OFLX alone were used as
controls). Cells are cultured in a 15 mL test tube (4 mL volume in total) with vigorous shaking (200 rpm, 37 °C) for about 18 hrs, samples were taken out at different time points (T 6h, T 12h and T 18h) for measuring the values of optical density (OD_{600 nm}). Results of MIC studies were plotted with Optical density (OD_{600 nm}) values versus the concentrations of conjugated OFLX molecules calculated from the conjugation ratio of each sized NPs and the concentration of nanoparticle solution and results shown are only for samples at T18h.
CHAPTER VI

CONCLUSION

In summary, this dissertation presents structural and functional studies of multidrug membrane transporters using molecular biology approaches, structural modeling, cutting-edge single NP plasmonic spectroscopy and dark-field optical microscopy and spectroscopy. Two representative MDR transporters, ABC (ATP-binding cassette, BmrA) and RND (resistance-nodulation-cell division, MexA,B-OprM) in gram-positive and gram-negative bacteria (*B. subtilis* and *P. aeruginosa*), are studied in real-time at single cell resolution.

In Chapter II, we fused EGFP with the N-terminus or C-terminus of BmrA to construct BmrA-EGFP fusion proteins and characterized their efflux functions in four strains of *B. subtilis* cells (Nt-BmrA-EGFP, Ct-BmrA-EGFP, BmrA, and ΔBmrA, *B. subtilis*) using fluorescence microscopy and spectroscopy. Fusion of ABC membrane transporters with EGFP offers the opportunity to visualize membrane transporters and study their interactions with substrates that lead to their translocations. Note that the fusion of ABC transporters with EGFP (BmrA-EGFP) may alter their efflux functions. Therefore, it is very crucial that we first characterize heir efflux functions prior to using them to probe molecular mechanisms of membrane transporters. The results show that BmrA-EGFP fusion proteins exhibit the similar efflux kinetics of BmrA and retain the efflux functions of BmrA. Modeled structures of the fusion proteins show a highly
flexible linker region connecting EGFP with BmrA, suggesting a minimal obstruction of EGFP to the BmrA.

In Chapter III, we studied the size-dependent efflux functions of BmrA-EGFP and BmrA using single plasmonic nanoparticle imaging probes and spectroscopy, and found size-dependent efflux kinetics of BmrA transporter. These two new stains of BmrA-EGFP offer the possibility for one to visualize BmrA transporter and study efflux function and mechanisms of BmrA.

In Chapter IV, using the similar approaches, we fused EGFP with MexB (subunit of MexA,B-OprM transporter) and characterized efflux functions of fusion protein (MexA-EGFP-MexB-OprM) using fluorescence spectroscopy. The results show that the EGFP fusion strain only partially retains the original efflux functions of MexA,B-OprM transporter. Structural analysis of fusion protein suggests possible steric hindrance of EGFP towards the conformational arrangements of MexA,B-OprM tripartite proteins, which led to the steric effect upon their efflux functions.

In Chapter V, we further functionalized Ag NPs with the antibiotic (ofloxacin), which is a representative substrate of MexA,B-OprM transporter in P. aeruginosa. We studied the antibacterial effects of ofloxacin alone (absence of the NPs), and various sized Ag NPs conjugated with ofloxacin. We found that the larger sized Ag NPs conjugated ofloxacin showed the lower MICs and higher inhibitory potency towards the bacterial growth in P. aeruginosa.

In summary, we have studied the structures and functions of two different types of MDR efflux pumps (ABC and RND) in both gram negative and gram
positive bacteria using fluorescence microscopy and spectroscopy, and single plasmonic microscopy and spectroscopy. The results offer new insights into the structural and functional studies of multidrug membrane transporters (efflux pumps), and have demonstrated the power of integrating new nanotechnology approaches with molecular biology techniques for better understanding of MDR.
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