Study of vancomycin derivatives on bio-conjugated activity and bio-optical imaging

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NANYANG
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**STUDY OF VANCOMYCIN DERIVATIVES ON
BIO-CONJUGATED ACTIVITY AND
BIO-OPTICAL IMAGING**

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SCHOOL OF PHYSICAL AND
MATHEMATICAL SCIENCE
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ABSTRACT

The glycopeptide antibiotic vancomycin (Van) is usually served as a last resort to effectively treat methicillin-resistant Gram-positive bacterial infections. It acts through the specific binding affinity to the C-termini D-Ala-D-Ala motif of peptidoglycan, which inhibits the biosynthesis of bacterial cell wall and leads to cell death. However, with overuse of antibiotic in clinic and animal feeds, bacteria having resistance to vancomycin emerged as a serious threat to public health, such as vancomycin-resistant *enterococci* (VRE). Their termini peptide sequence is mutated from D-Ala-D-Ala to D-Ala-D-Lac, which results in around 1000-fold decrease in binding affinity to vancomycin.

This research dissertation focuses on the study of vancomycin and its derivatives on molecular interaction, photodynamic antibacterial activity and bacterial Raman imaging. Chapter 2 describes the investigation of molecular interactions between vancomycin and bacterial cell wall analogs in various solutions based on fluorescence correlation spectroscopy (FCS) and molecular dynamic (MD) simulation. This study clearly demonstrates that the components constituting the buffer solution have a profoundly disturbing effect on the complex formed between low-affinity drug resistant bacterial cell peptide and vancomycin. These results facilitate the fully understanding of the structure and activity of vancomycin towards bacterial cell wall peptide analogs, which is significant to design novel drug candidates against drug-resistant bacterial strains.

In Chapter 3, a simple and specific multifunctional divalent vancomycin derivative is designed and synthesized through conjugating two vancomycin moieties with porphyrin. This divalent Van-porphyrin not only exhibits excellent photodynamic antimicrobial activity towards vancomycin-sensitive and vancomycin-resistant *enterococci* (VRE) strains but also could be served as a promising fluorescent probe for living bacterial imaging.

Chapter 4 describes the study on bacterial Raman imaging based on a vancomycin modified surface enhanced Raman scattering (SERS) bio-tag. This SERS bio-tag is employed to specific bind to Gram-positive bacterial strains *via* multivalent interaction between bacterial cell walls and polyvalent vancomycin affinity groups present on the surface of SERS nanoparticles. Bacterial Raman imaging results demonstrate that this SERS bio-tag possesses promising possibilities for a substituent of fluorescent probe in living bacterial imaging.

Abbreviations

Å	Angstrom
AgNPs	silver nanoparticle
Asn	Asparagine
br	broad
CD	Circular Dichroism
CFU	colony forming units
d	doublet
D-Ala-D-Ala	D-Alanine-D-Alanine
D-Ala-D-Lac	D-Alanine-D-Lactate
DAP	meso-diaminopimelic acid
dd	doublet of a doublet
DLS	dynamic light scattering
DPG	dihydroxyphenylglycine
ESP	electrostatic potential
FCS	fluorescence correlation spectroscopy
fs	femtosecond
GA	glycopeptides antibiotics
GFP	green fluorescent protein
GlcNAc	N-acetyl glucosamine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPG	hydroxyphenylglycine
ITC	isothermal titration calorimetry
K_a	association constant
K_d	dissociation constant
Leu	Leucine
m	multiplet
MD	molecular dynamic
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MurNAc	N-acetyl muramic acid
nm	nanometer
ns	nanosecond
o	ortho
PACT	photodynamic antimicrobial chemotherapy
PBPs	penicillin-binding proteins
PBS	phosphate buffered saline
PG	peptidoglycan
Phe	Phenylalanine
QDs	quantum dots
RMSD	root-mean-square deviation
ROS	reactive oxygen species
RP-HPLC	reversed phase high performance liquid chromatography

p	para
PS	photosensitizer
ps	picosecond
s	singlet
SERS	surface enhanced Raman scattering
SPR	surface plasmon resonance
TEM	transmission electron microscopy
Tyr	Tryptophan
UDP-GlcNAc	UDP-N-acetylglucosamine
UV-Vis	ultraviolet visible spectroscopy
Van	vancomycin
VRE	vancomycin-resistant enterococci
δ	NMR chemical shift in ppm

Chapter 1

Introduction

1.1 Bacteria:

Bacteria, a large realm of single-celled prokaryote microorganisms, are close related to human life. Approximately, 100 trillions bacterial cells exist in human's bodies (eg. skin and digestive tracts), most of them are rendered harmless to human being, and a few are even beneficial to good health.¹ However, some species of them cause infectious diseases and death, seriously threatening public health. Therefore, fully understanding the structures and biological behaviors of pathogenic bacteria is fundamental to their clinic treatments.

1.1.1 Structure of bacterial cell wall

Most bacteria are mainly composed of nucleoid (DNA), ribosomes, cell membrane and cell wall. Among them, cell wall plays an important role to maintain cell shape and protect against osmotic lysis. Two general categories of bacterial cell walls, Gram-positive bacteria and Gram-negative bacteria, were first distinguished over a century ago by Hans Christian Gram based on their different retention of crystal-violet dye.² As shown in Figure 1.1, The Gram-positive cell wall is composed of a thick, mesh-like peptidoglycan (PG) sheath outside of the cytoplasmic membrane, providing mechanical support to the cell. Teichoic acids and lipoteichoic acids are linked to and embedded in the multilayer peptidoglycan.^{3,4} While, Gram-negative cell wall contains an outer membrane linked by lipoproteins to peptidoglycan, which is

thin, single-layered and located within the periplasm between the outer and inner membranes.^{5,6} Porins embedded in the outer membrane are transport channels for small hydrophilic molecules to across the membrane.⁷ Compared to Gram-negative bacteria, Gram-positive bacteria typically lack the outer membrane and have a much thick peptidoglycan layer that enable to retain crystal-violet dye.

Figure 1.1. Structure of Gram-positive (a) and Gram-negative bacterial cell walls (b). Gram-positive cell walls are composed of a thick (20–80 nm), multilayered peptidoglycan sheath that includes embedded teichoic and lipoteichoic acids; Gram-negative cell walls include an outer membrane that surrounds a thin (1–7 nm, depending on measurement technique) peptidoglycan layer, with a periplasmic space between the inner and outer membranes.⁷

1.1.1.1 Molecular structure of peptidoglycan

Peptidoglycan is a layer of rigid exoskeleton, conferring strength and rigidity to bacterial cell, resisting intercellular osmotic pressure, maintaining its cell shape, allowing growth and division. As its name implies, the basic structure of peptidoglycan is composed of stiff polysaccharide chains and flexible peptide bridges. Linear polysaccharide strand is parallel and typically made of repeating residues including the carbohydrates N-acetyl glucosamine (commonly referred to as GlcNAc) and N-acetyl muramic acid (MurNAc).⁸ The lactyl group on MurNAc is substituted with a tetra-peptide moiety, which is cross-linked each other, either directly or through another short penta-peptide. Compared to consistent composition of polysaccharide, the backbone of tetra-peptide in peptidoglycan is not conserved in all bacteria, in the company of some variations in the third amino acid: most Gram-positive bacteria employ L-lysine, named as Lys-type peptidoglycan, in which penta-peptide including five glycine residues links the tetra-peptide from the L-lysine residue to the terminal D-alanine residue of another tetra-peptide; however, meso-diaminopimelic acid (DAP) is commonly utilized instead in Gram-negative bacteria and Gram-positive *bacilli* (DAP-type peptidoglycan), usually directly cross-linked each other without interpeptide bridges.⁹ Figure 1.2 shows the detail structures of two classes of peptidoglycans and how the polysaccharide strands and peptide chains come together to form lattice-like peptidoglycan, imparting strength and stability to the bacterial cell wall.

Figure 1.2. The fundamental structure units of peptidoglycan. (a) DAP-type peptidoglycan, in which m-DAP links to D-Ala without peptide bridges; (b) Lys-type peptidoglycan, where a penta-peptide $(Gly)_5$ is involved into the linkage between L-Lys and D-Ala.⁹

1.1.1.2 Biosynthesis of peptidoglycan

The biosynthesis of peptidoglycan is believed to occur in three distinct stages. Figure 1.3 shows the detail biosynthesis pathway. The first stage occurs in the cytoplasm and initiates with conversion of Uridine diphosphate -N-acetylglucosamine (UDP-GlcNAc, **1**) into UDP-N-acetylmuramyl-pentapeptide (**3**).¹⁰⁻¹² The initial step is catalyzed by MurA enzyme, which transports an enolpyruvyl group onto the 3-hydroxyl group of UDP-GlcNAc. Subsequently, MurB reductase mediates an NADPH-dependent reduction to converse of the enol group into the lactyl moiety and produces UDP-N-acetylmuramic acid (**2**). ATP-dependent ligase MurC, D, E, F introduce the amide bond L-Ala, D-Glu, L-Lys (Gram-positive bacteria) or m-DAP (Gram-negative bacteria)¹³ and D-Ala-D-Ala, in succession, onto the C(3)-lactyl

moiety to provide UDP-MurNAc-pentapeptide (3).¹⁰⁻¹²

The second stage of peptidoglycan biosynthesis involves two steps. The initial one involves the coupling of UDP-MurNAc-pentapeptide and (3) to a membrane anchored lipid (C55) carrier to produce undecaprenylpyrophosphoryl-MurNAc-pentapeptide (lipid I, 4), which is accomplished through a Mra Y enzyme mediated pyrophosphate exchange reaction and concomitant with the ejection of UMP. Secondly, MurG enzyme catalyzes the transformation process of GlcNAc from UDP-GlcNAc to the C(4)-hydroxyl group of the lipid I, obtaining the final monomeric intermediate in the cell wall biosynthesis cascade, lipid II (5).^{10,11,14} Once lipid II is formed, it is flipped to the external face of the cell membrane, which is called translocation process.

The third and final stage involves the elongation of glycan strains and the cross-link of pentapeptides, which are facilitated by a group of membrane-bound enzymes named penicillin-binding proteins (PBPs), including transglycosylases and transpeptidases.^{15,16} On the extracellular surface of the bacterial cell membrane, transglycosylases are involved to bond the disaccharide moiety of the precursor and existing polysaccharide strands *via* catalyzing the formation of new β -1,4-glycosidic bond between them. Linear and parallel polysaccharides with repeating GlcNAc and MurNAc units are generated by means of transglycosylation processes. Subsequently, penta-peptide subunits, protruding perpendicularly from the sugar backbone, are crosslinked in a pair-wise manner through transpeptidases-catalyzed amide bonds formation between the terminal amino group of the lysine or DAP residue and the penultimate D-Ala residue of an adjacent chain.¹⁷ The generated amide bond is

concomitant with loss of the terminal D-Ala residue.^{15,16}

Figure 1.3. The biosynthesis pathway of peptidoglycan. It involves three different stages. Stage one and two occur at cytoplasm which is inside the bacterial cell membrane. While, stage three is initiated outside the cell membrane.¹⁷

1.2 Antibiotic

Although bacteria cause serious diseases and threaten public health, human have found a powerful weapon, antibiotic, to combat them. To date, antibiotic drugs play an important role in bacteria caused infection therapy due to the highly efficient killing of pathogenic bacteria.

1.2.1 Peptidoglycan targeted antibiotics

Peptidoglycan has proven to be an excellent target for antibacterial actions since it is unique to bacteria and highly significant for cell survival.¹⁸ Figure 1.4 shows some antibiotics (highlighted in red) targeted to peptidoglycan biosynthetic pathway. In fact, most of them are derived from natural-products generated by bacteria themselves.¹⁹ Although three biosynthesis stages are all available for antibiotic agents to target, majority of them inhibit the final steps of peptidoglycan biosynthesis. The possible reason is that these steps occur outside of cell membrane and antibiotic drugs are easy to target the extracellular enzymes involved, avoiding the cell-membrane penetration.

Figure 1.4. The peptidoglycan biosynthetic pathway showing sites of actions of natural product inhibitors.¹⁸ Fosfomycin and D-cycloserine target to the first stage; uridylpeptides, ramoplanin, amphomycin and bacitracin acts on the second stage; the third stage involves the inhibitions from penicillin beta-lactams, moenomycin and vancomycin glycopeptides.

1.2.2 Classification of antibiotics targeted to stage three in peptidoglycan synthesis

Antibiotics that inhibit the final stage are categorized into three basic classes with respect to mechanisms of actions.²⁰ As shown in Figure 1.5, the first class, including β-lactams, carbapenems, and cephalosporins, prevents the procedures of transpeptidations through selectively binding to the active sites of transpeptidases. The second class of antibiotics inhibits polysaccharide elongations by binding to bacterial transglycosylases and terminating the steps of transglycosylations.

Moenomycin is the typical member of this class, which is only used in animal feeds rather than human beings due to its poor physical properties. Unlike to the enzyme-targeted activity of the first and second classes, the third class of antibiotics acts on the substrates of transglycosylases and transpeptidases.¹³ It comprises several families, such as ramoplanin, lantibiotic and glycopeptides, in which glycopeptide antibiotics (GA) are probably the most famous in this class.¹³

Figure 1.5. Prototypical member of three classes of antibiotics inhibiting stage three in peptidoglycan assembly: β -lactams, moenomycin, and glycopeptides.¹³

1.2.3 Glycopeptide vancomycin

Glycopeptide antibiotics (GA) are the well-known of these substrate-binding antibiotics. Vancomycin (Van), as the archetypical member of GA family, is one of the most studied antibiotics. It also serves as a model system to investigate the mechanistic action of glycopeptides since other GAs kill bacterial cells through the same mechanism of action as vancomycin, which is to block the substrates of transpeptidases in the peptidoglycan biosynthesis.²¹ Vancomycin is a natural product

isolated from *Amycolatopsis orientalis* in 1953 by Edmund Kornfeld.^{16,22} It is effective against Gram-positive bacteria rather than Gram-negative ones, because the outer membrane in Gram-negative bacteria plays a role of permeability barrier to keep the glycopeptide antibiotics from reaching their targets in the peptidoglycan. In many countries, vancomycin has been approved for use in clinic. It was initially only used with patients allergic to beta-lactam antibiotics. However, with the emergency of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin has become the last resort to treat the life-threatening infections caused by bacteria resistant to beta-lactam antibiotics.^{23,24}

1.2.3.1 Structure of vancomycin

The structure of vancomycin was put forth through by Harris and Williams labs in 1982.^{25,26} Figure 1.6 shows the molecular structure, which can be divided into two parts: a disaccharide and a homologous heptapeptide scaffold. The heptapeptide outlined in red is composed of Leu1, Asn3 and five aromatic components Tyr2, Phe4, Phe5, Tyr6 and Phe7. These residues undergo extensive oxidative crosslinking and form the backbone of vancomycin.²⁷ Phe4, 5 7 are nonproteinogenic phenylglycines, which are 4-hydroxyphenylglycine (HPG) for residues 4, 5 and 3,5-dihydroxyphenylglycine (DPG) for residue 7, respectively. Similarly, Tyr 2 and 6 are also modified tyrosines, with chlorine at the meta position of the aromatic ring and an hydroxyl group substituted at the benzylic carbon of the side chain. The disaccharide is D-glucosyl-2,1-D-vancosamine, which is linked through C1 position of glucose to

the phenolic oxygen of Phe4 residue in the heptapeptide scaffold. The vancosamine is deoxy sugar, with methyl and amino substituted groups, which mediate the molecular recognitions between vancomycin and target groups.

Figure 1.6. The structure of vancomycin. It is composed of two components: disaccharide (vancosamine and glucose, purple) and heptapeptide scaffold (red). The seven modified peptide residues are highly crosslinked to form scaffold of vancomycin.

1.2.3.2 Antibacterial mechanism of vancomycin

Although vancomycin was firstly discovered in 1953, its general mechanism has not been made clear until 1970. The initial study on its mechanism of antibacterial action was reported by Strominger and co-workers in 1965.²⁸ This work was a masterpiece since the biosynthesis of peptidoglycan was still not understood. Subsequently, a series of research works from Perkins's group verified that vancomycin functions through binding to the extracellular peptidoglycan precursor

terminus N-acyl-D-Ala-D-Ala.^{29,30} Shortly after that, Williams *et al.* employed NMR to demonstrate that the binding interaction is formed through five hydrogen bonds between D-Ala-D-Ala dipeptide and the heptapeptide backbone of the vancomycin (Figure 1.7).

Figure 1.7. Complexation of vancomycin with N-acyl-D-Ala-D-Ala terminal peptide. Five hydrogen bonds are formed between the underside of the glycopeptide antibiotic and the N-acyl-D,D-dipeptide moiety.¹³

Upon binding to D-Ala-D-Ala, vancomycin takes action to sterically block transglycosylation and/or transpeptidation processes, preventing the essential cross linking of peptidoglycan and resulting in weak peptidoglycan layers. Thus, bacterial cells become susceptible to lysis because of changes in osmotic pressure. Figure 1.8 demonstrates the mechanism of vancomycin antibacterial action in detail.

Vancomycin: Mechanism of Action

Figure 1.8. The antibacterial mechanism of vancomycin. Bacterial cell walls function as a barrier to be resistant to osmotic pressure, enabling cell growth and survival. Once vancomycin bonds onto the terminal dipeptides, the biosynthesis process of peptidoglycan is sterically blocked, resulting in susceptibility to osmotic pressure and eventual cell death.

1.3 Antibiotics resistance

Antibiotic resistance is a type of drug resistance where bacteria show the capability to survive the effects of an antibiotic. Bacteria become resistant to antibiotics are due to the resistant genes resided on plasmids, which enable drug-resistant properties easily spread through gene dissemination or plasmid exchange between bacteria. The

prevalence of resistant bacteria is mainly due to the widespread use of antibiotics both inside and outside of medicine. Natural selection plays an essential role in the development of antibiotic resistance. As an environmental stress, antibiotic kills the bacteria without mutations. Only resistant bacteria can survive and reproduce next generations with the same antibiotic resistant trait.

1.3.1 Occurrence of vancomycin-resistant *enterococci* (VRE) strain

With overuse of vancomycin in clinic, in company with that of related glycopeptide antibiotics in animal feeds, vancomycin-resistant Gram-positive bacteria have emerged.³¹ The first vancomycin-resistant *enterococci* (VRE) strain was isolated from patients in an American hospital in 1987.³² Since then, the prevalence of VRE strains have extended from the local to the global; The percentage of VRE reported among the bacterial infections sharply increased in decades^{24,33} (Figure1.9). Besides the worldwide spread of VRE strains themselves, the transfer of their glycopeptide resistance genes is another major reason for the increased occurrence of vancomycin resistance in the world. Noble and Sanyal *et al.* have confirmed that the resistance genes can be transferred from *enterococci* to methicillin resistant *Staphylococcus aureus* or *S. epidermidis*.^{34,35} Since the first clinic vancomycin-resistant *Staphylococcus aureus* (VRSA) was described in 1998,³⁶ over ten cases of VRSA were reported and confirmed in America by 2002.³³ Meanwhile, the cases of VRE and VRSA reported in the whole world remain increasing with a very high rate every year, which accompany with the rising number of deaths from bacterial infections. The

emergence and prevalence of all these drug resistant bacterial strains mean a serious global health threat, which strongly requires new approaches to treat infections caused by them.

Figure 1.9. Occurrence rate of VRE in the United States³⁷⁻³⁹, North America (left) Latin America, Europe and Asia-Pacific region. (right) (Sentry program, 1997-2000)^{33,40}

1.3.2 Classification of VRE

VRE strains fall into three major classes with respect to distinct clinical phenotypes: VanA, VanB and VanC, which confer various degrees of drug-resistance to vancomycin. VanA and VanB phenotypes show around 1000-fold increased resistance to vancomycin.^{13,41,42} Besides, VanA isolates also exhibit highly resistance to another glycopeptide antibiotic, teicoplanin.⁴³ This high-level resistance is caused from five *van* genes, VanRSHAX⁴⁴⁻⁴⁶ (Figure 1.10). Compared to VanA and VanB, only around 1 log increased in resistance is found in VanC VRE isolates, which have not been prevalent in humans.⁴²

Figure 1.10. Scheme of the five genes VanRSHAX cassette in VanA and VanB phenotypes of VRE. These genes encode VanRSHAX proteins to catalyze the reprogramming of peptidoglycan termini from N-acyl-D-Ala-D-Ala to N-acyl-D-Ala-D-Lac.¹³

1.3.3 Mechanism of VRE resistance

Extensive research works done by Walsh and co-workers illustrated that VanA and VanB VRE strains involve the reprogramming of the peptidoglycan terminus from D-Ala-D-Ala to D-Ala-D-Lac.^{44,47,48} Mutating this peptide termini does not affect the quality of the peptidoglycan layer or its stability to crosslink the polypeptide strands. However, it could decrease the affinity of vancomycin for bacterial cells by a factor of 1000,⁴⁹ thereby, rendering the antibiotic ineffective against VRE strains. D. L. Boger *et al.* have provided detailed experimental data to verify the conclusion that the complex of vancomycin with N-acyl-D-Ala-D-Lac not only loses the central hydrogen bond, a characteristic of the D-Ala-D-Ala complex, but also suffers from a repulsive lone pair interaction between the carbonyl group of residue 4 and the D-Ala-D-Lac ester oxygen⁵⁰ (Figure 1.11). Due to the loss binding between

vancomycin and bacterial cell walls, the biosynthesis of peptidoglycan cannot be terminated, rendering the antibiotic ineffective against VRE strains.

Figure 1.11. Loss of the one hydrogen bond in the complex between vancomycin and D-Ala-D-Lac, in comparison of D-Ala-D-Ala, and the oxygen-oxygen lone pair repulsion account for a 1000-fold decrease in binding affinity.¹³

1.3.4 Molecular modeling between vancomycin and bacterial cell wall analogs

Given the therapeutic and industrial importance of vancomycin, its binding to the modified precursors has become a molecular modeling as a tool for designing next-generation drugs to fight the evolving drug resistant bacterial strains. Therefore, in the past few decades, detailed understanding of the interactions between vancomycin and the peptide ligands has become the subject of considerable experimental and theoretical investigations. Previous reported methods have yielded ensemble average data of the molecular recognition in various buffer solutions.

Table 1.1. Selective research works on the interaction between vancomycin and bacterial cell wall mimicking peptide ligands.

Research Groups	Van/ D-Ala-D-Ala	Van/ D-Ala-D-Lac	Buffer systems	Detection methods
	K_d^a	K_d^a		
John H. Griffin	$1.1 \pm 0.4 \mu\text{M}$	$1800 \pm 65 \mu\text{M}$	10 mM HEPES; 6 mM NaCl; 25°C pH 7.0	Fluorescence quenching titration ⁵¹
R. F. Pratt	$3.3 \mu\text{M}$	-	0.1 M Phosphate buffer; 25°C; pH 7.0	UV-Vis; Stop-flow; Circular dichroism (CD) Spectroscopy ^{52,53}
	$0.7 \mu\text{M}$	$3030.3 \mu\text{M}$	0.1 M Phosphate buffer	Competitive binding based fluorescent titration ⁵⁴
Geroge M. Whitesides	$1.6 \mu\text{M}$	-	20 mM Phosphate buffer; 25°C; pH 7.0	Isothermal titration calorimetry (ITC) Titration ⁵⁵
	-	$1700 \mu\text{M}$	0.2 M Phosphate buffer	Isothermal titration microcalorimetry ⁵⁶
Jonathan A. Ellman	$1.3 \mu\text{M}$	-	10 mM HEPES; 6 mM NaCl; 25°C; pH 7.0	Fluorescence titration; Surface plasmon resonance (SPR) ⁵⁷
J. N. Hobbs	$2.4 \mu\text{M}$	$1960 \mu\text{M}$	20 - 75 mM Phosphate buffer; pH 6.7 - 7.0	Affinity capillary electrophoresis ⁵⁸
	$2.4 \mu\text{M}$	$2439 \mu\text{M}$		Affinity capillary electrophoresis ⁵⁹
Dale L. Boger	$1.7 \mu\text{M}$	$2326 \mu\text{M}$	20 μM sodium citrate	UV-Vis ⁵⁰

^a: Dissociation binding constant

As shown in Table 1.1, the binding affinities between vancomycin and the ligands D-Ala-D-Ala and D-Ala-D-Lac in buffer solutions have been studied based on several

methods including UV-difference spectroscopy,^{50,52,53} fluorescence,^{51,54,57} affinity capillary electrophoresis^{58,59} and isothermal titration microcalorimetry.^{55,56} Due to the different experimental techniques and analysis methods used, there exists inconsistency between the various reported binding constant values. Perkins and Pratt *et al.* have found that the degree of association between vancomycin and Ac₂KDADA can be affected by solvent properties such as pH, ionic strength and the type of solvents used.^{52,60} Although the environmental effects have been characterized in extensive studies on the binding between vancomycin derivatives and bacterial cell wall peptide precursors, the exact nature of the discrepancy in the equilibrium binding constant values reported so far is still unclear.^{56,61} In fact, buffer solutions have been proved to disturb the coupling of ligands and receptors in many biological systems.^{62,63} Therefore, it becomes necessary to explore the detailed information on the effects of the various components that make up the buffer (*e.g.*, phosphate ion in phosphate buffer etc.) on the association between vancomycin and peptide analogs.

Fluorescence correlation spectroscopy (FCS) is a direct spectroscopic technique to analyze the diffusion of an emitting molecule as it diffuses in the excitation volume of a confocal microscope. The binding constants of two interacting species could be quantified based on the difference of diffusion behaviors between a freely diffusing emitting molecule and when it binds to a target. Figure 1.12 shows the principles of FCS, where a protein tagged with green fluorescent protein (GFP) interacts with its ligand to form a protein complex. The autocorrelation curves obtained from FCS measurements exhibit the different diffusion behaviors of individual proteins and the

complex. As a simple and powerful tool, FCS has been widely used to analyze molecular interactions in aqueous solution with single molecule sensitivity, such as the inclusion/exclusion behaviors between pyronines and cyclodextrin⁶⁴, binding affinity of antigen/antibody⁶⁵ and equilibrium complex formation of transferrin ligand and its receptor⁶⁶. FCS has some significant advantages over traditional approaches, such as UV-difference spectroscopy and conventional fluorescence techniques. For example, FCS does not involve an environment-sensitive chromophore to alter its photophysical properties upon complex formation between ligands and receptors. Also, the relative low concentration of sample in FCS examinations prevents their self-aggregation or dimerization frequently occurred at high concentration. In our study, the bacterial cell wall peptide analogs, *N,N'*-acetyl-L-Lys-D-Ala-D-Ala (AcK(NH₂)DADA) and *N,N'*-acetyl-L-Lys-D-Ala-D-Lac (AcK(NH₂)DADL), are labeled with ATTO 655 which is photostable and commonly used near-infrared fluorescent dye in FCS studies. The influences of buffer systems on their intrinsic binding are systematically investigated using both single molecule fluorescence correlation spectroscopy (FCS) and molecular dynamics (MD) simulations, which are fully described in Chapter 2.

Figure 1.12. Principles of fluorescence correlation spectroscopy (FCS). (a) When a fluorescent labeled protein diffuses within the confocal volume, GFP serves as the attached fluorophore to emit photons. Compare to individual protein (left), protein bounded complex (right) possesses a lower diffuse and resides in the confocal volume for more time. (b) From FCS measurements of the corresponding fluctuations in fluorescence intensity over time, an autocorrelation curve can be calculated.⁶⁷

1.3.5 Novel drug design for vancomycin resistant bacteria

Since vancomycin resistant bacteria have emerged as important pathogens in clinic, seriously threatening public health, there is an urgent need to develop novel alternative approaches to kill these drug resistant bacterial pathogens. One strategy is based on a model system involving vancomycin analogues to enhance their binding affinities to the cell wall of resistant bacteria, thus killing them by the same mechanism against sensitive strains. This model involves simultaneous multivalent/polyvalent interactions between two biological entities, which are much stronger than corresponding monovalent interactions.^{68,69} The possible principle of

multivalency is proposed by Whiteside and co-workers. In multivalent system, the gains in free energy of association may be offset by the loss in conformational entropy of the linkers upon binding, which results in tight binding pairs.³³ Some criteria are also outlined to give large binding constants: (1) rigid receptors and ligands; (2) conformational inflexible linkers; (3) a large hydrophobic component.³³ On the basis of this multivalency theory, well-defined multivalent vancomycin derivatives should offer an increased binding affinity to the multiple copies of D-Ala-D-Lac dipeptidic termini presented on the cell surfaces of vancomycin resistant bacteria. Up to now, several research groups have devised a variety of vancomycin dimmers or oligomers to explore their antimicrobial properties against resistant bacterial strains. As shown in Figure 1.13, Griffin *et al.* employed carboxamide linkers to form vancomycin dimmers, which exhibit great potency against VRE organisms.^{51,70,71} Based on the data of *in vitro* activities, it seems that the potency of vancomycin dimmers against VRE decreased with the increase of the flexibility of the linkers, which is agreed with the principles of multivalency proposed by Whitesides. Nicolaou and co-workers synthesized a library of vancomycin dimmers through linking the vancosamine amino groups using olefin metathesis reaction and disulfide formation. These divalent vancomycins afforded several highly effective antibiotics against both VRE strains and vancomycin-intermediate *Staphylococcus aureus* (VISA)⁷²⁻⁷⁴ (Figure 1.14). In Xu's group, a derivative of cisplatin was served as a rigid square planar linker to form dimeric vancomycin derivatives. The combination of vancomycin and the metal complex linker offered special geometry, structure rigidity and stability to minimize

the loss of conformational entropy, producing improved activities against VRE strains⁷⁵ (Figure 1.15). Besides divalent systems of vancomycin, Arimoto *et al.* also designed polyvalent oligomeric vancomycin and evaluated its enhancement of potency against VRE strains⁷⁶ (Figure 1.16).

Figure 1.13. Synthesis routs and structures of bis(vancomycin) carboxamides. These compounds not only retain *in vitro* activity against vancomycin-susceptible bacteria but also exhibit substantially enhanced potency against VRE strains.^{51,70}

Figure 1.14. The molecular structure of vancomycin dimers with the vancosamine amino groups linkages, which show high antibacterial activities against vancomycin-susceptible and vancomycin-resistant bacteria.⁷³

Figure 1.15. The structure of divalent and monovalent derivatives of vancomycins based on a rigid metal complex. These metal complex linkers have special geometry, structural rigidity and stability, facilitating to minimize the loss of conformational entropy in the construction of multivalent antibiotics.⁷⁵

Figure 1.16. The structures of the monomer and oligmeric vancomycin. The oligmers in **18a** have more repeating units than that of **18b**, which shows the enhancement of potency against VRE bacterial strains.⁷⁶

Although covalently linked dimmers or oligomers of vancomycin display high binding affinity with vancomycin resistant bacteria, the increased binding affinity may not always lead to considerable activities with effective minimum inhibitory concentration (MIC) against VRE bacterial strains.^{77,78} In order to address this issue, photodynamic antimicrobial chemotherapy (PACT) was introduced and serves as an alternative treatment strategy against VRE bacterial infections.⁷⁹ This emerging technology involves the utilization of light-activated agents, known as photosensitizer (PS), which could be activated by visible light to generate reactive oxygen species (ROS, e.g. singlet oxygen ($^1\text{O}_2$)).⁸⁰ These reactive oxygen species can damage plasma membranes and lead to cell death.⁸¹ Figure 1.17 shows the mechanism of action in detail. Briefly, upon irradiation with light, photosensitizer (PS) undergoes a transition from the ground state to short-lived singlet state ($^1\text{PS}^*$), subsequently following an intersystem crossing to a long-lived triplet state ($^3\text{PS}^*$). The triplet state reacts with

endogenous oxygen to generate singlet oxygen species following two alternative kinds of pathways.^{82,83} Type I involves the generation of radicals *via* charge transfer from $^3\text{PS}^*$ to surrounding substrates. These radicals react rapidly with oxygen, producing highly reactive oxygen species (superoxide, hydroxyl radicals, hydrogen peroxide). In a Type II pathway, singlet oxygen is generated by direct energy transfer from the excited $^3\text{PS}^*$ to molecular oxygen. The generated ROS, both from Type I and Type II, can rapidly offer localization dependent oxidative damage to bacterial cell walls, subverting their function and eventually causing cell death.

Figure 1.17. Mechanism of photodynamic antimicrobial therapy. Visible light activates the given photosensitizer (PS) from the ground state to the excited form $^3\text{PS}^*$, which can follow two alternative kinds of pathways to generate reactive oxygen species (ROS). Electron transfer to O_2 can produce superoxide, hydrogen peroxide and hydroxyl radicals (Type I); In Type II of reaction, energy transfer to O_2 results in the formation of singlet oxygen ($^1\text{O}_2$), all of which are very cytotoxic to the pathogens due to the oxidation damage caused.^{81,84}

So far, considerable research works have identified that PACT is highly effective against a variety of Gram-positive and Gram-negative bacteria.^{80,85,86} In order to minimize side effects and improve the efficiency of PACT in clinics, affinity ligands are widely used as targeting groups for bacterial infections. Previous reported affinity ligands, including antibodies,⁸⁷ protein cage,⁸⁸ polypeptide,⁸⁹ nanoparticles,⁹⁰ and bacteriophage,⁹¹ have been proved to enable lethal photosensitizers successfully target to antibiotic-resistant bacteria. Although great efforts have been performed to improve the efficiency of PACT in virtue of various binding affinity groups, some drawbacks still exist, such as complicated and tedious manipulation, difficulty in synthesis, self-aggregation and possible immunogenicity.⁸⁷⁻⁹¹ Therefore, it remains necessary to develop simpler and economical novel targeting molecules capable of specifically directing photosensitizers to drug resistant bacteria.

In Chapter 3, a commonly used photosensitizer, porphyrin was conjugated with two vancomycin moieties to serve as a simple and novel phototherapeutic reagent against drug resistant bacterial strains, in which rigid porphyrin linker supplies entropically enhanced binding and steric hindrance necessary for multivalent interactions between the disubstituted vancomycin and VRE strains.

1.3.6 Vancomycin based optical bacterial imaging

Bacteria, especially drug resistant bacteria, cause numbers of diseases through an extremely complex infection process and become a serious threat to human health. Optical bacterial imaging provides a straightforward method to investigate biological

behaviors of bacteria and establish disease models to study bacterial infection.⁹² Meanwhile, this emerging technology also brings up a way with high sensitivity and efficiency to identify microbial contamination and track infection process⁹³⁻⁹⁵, further facilitating medical diagnosis in clinic. During the development of bacterial imaging technology, exploration of suitable reporters remains an extremely critical challenge. One approach is to employ the genetically encoded bacteria that produce luciferase or green fluorescent protein (GFP). These protein reporters and their analogs successfully provide insight into many biological activities at the molecular and cellular levels.⁹⁶ However, their applications have been limited due to the complicated, time-consuming and expensive transgenic procedures. Therefore, bio-tags with an affinity ligand and a reporter group are anticipated to emerge as an alternative strategy.

Vancomycin group antibiotics have been extensively used as affinity groups for Gram-positive bacteria due to its strong and specific binding interaction with peptidoglycan of bacterial cell walls. Considerable studies have been performed to investigate the architecture of bacterial cell walls based on the use of fluorescent labeled vancomycin derivatives. As shown in Table 1.2, the most extensive used imaging materials are organic fluorescence dyes.^{97,98 99,100}

Table 1.2. Research works on the architecture of bacterial cell walls based on fluorescent vancomycin analogs.

Research Groups	Probes	Bacterial strains	Applications
Jeff Errington ⁹⁷	Fluorescein labeled vancomycin	<i>B. Subtilis</i> ^a	explore nascent peptidoglycan synthesis and reveal the ways to control cell morphogenesis in Gram-positive bacteria.
David Z. Rudner and Suzanne Walker ⁹⁸	Fluorescein or BodipyFI labeled vancomycin or ramoplanin;	<i>B. Subtilis</i> ^a	image the peptidoglycan to determine the genes that control the biosynthesis
Mariana G. Pinho ⁹⁹	BodipyFI labeled vancomycin	<i>S. aureus</i> NCTC8325-4 ^a ; COL ^a ; JH1 ^a <i>S. aureus</i> VM50 ^b ; JH9 ^b	investigate the mechanism of vancomycin resistance and establish the molecular models of vancomycin resistant <i>S. aureus</i> .
Yves F. Dufrene ¹⁰⁰	BodipyFI labeled vancomycin	<i>L. lactis</i> NZ3900 ^a ; <i>L. lactis</i> MD003 ^b	explore the architecture and assembly of peptidoglycan during the cell cycle of Gram-positive bacteria.

^a: vancomycin susceptible strain; ^b: vancomycin resistant strain

As nonradioactive tagging materials,¹⁰¹ fluorescent dyes have shown great potential as imaging probes *ex vivo*, both for cellular imaging and for biosensor applications. However, several instinct drawbacks exist with fluorescent materials when they are employed *in vivo* biological imaging. Firstly, photobleaching is a ubiquitous problem with all fluorophores,¹⁰² even though the use of quantum dots is associated with minimal photobleaching.¹⁰³ Secondly, multiplex detections in multiple labeling with fluorescent molecules are restricted due to limited numbers of fluorescent agents and their broad emission profiles.¹⁰⁴ Thirdly, autofluorescence emanating from superficial tissue layers affects the sensitivity of fluorescence, offering a higher signal-to-noise

ratio.¹⁰⁵ One of possible strategies to address these issues is to use surface enhanced Raman scattering (SERS) probes as a substitute of fluorescent materials.¹⁰⁶⁻¹¹⁰ SERS is a surface-sensitive technique that capitalizes on the enhancement of a Raman signal generated by molecules adsorbed onto the surface of nano-roughened noble metal. Raman signal highly increases due to the dramatic increase of electromagnetic field provided by plasmon surface.¹¹¹ The enhancement can be as high as 10^6 - 10^{14} folds, allowing SERS probes to detect pico- to femtomolar amounts of biomolecules, which offers two or three orders of magnitude higher sensitivity in comparison to fluorescence.¹¹²⁻¹¹⁴ SERS tags exhibit inherent resistance to photo-bleaching and offer narrow Raman peaks to avoid spectral overlapping, overcoming the inherent weaknesses of fluorescence imaging. Multi light sources, especially near infrared light, could be used to excite SERS probes, allowing light penetrate to deep tissue and making it ideal for *in vivo* imaging.¹¹⁵⁻¹¹⁸ Thus, SERS tags have been received great attentions to serve as a class of emerging labels for biological systems.

Currently, SERS tagging approaches could be divided into two main categories: direct detection methods (detection of target SERS peak, also named label free) and indirect detection methods (detection of Raman-label compounds).¹¹⁹ During the past decades, considerable efforts have been devoted to the label free SERS-related research works, in which SERS signals were generated through adsorbing intact biological species onto roughened metal nanoparticle surface. For example, Kneipp *et al.* applied SERS for intracellular cell imaging through injecting gold or silver nanoparticles into a single cell;^{108,120} A similar label-free microarray system was also

developed by Haisch and his colleges for *in situ* detection of microorganisms.¹²¹ However, as a readout approach, its application for bioassays is limited by the general disadvantages, such as lack of sensitivity, difficult involving in non-purified samples, limitation of dynamic range and highly complex SERS signals.¹²² Thus, indirect SERS detection approaches were developed through introducing Raman-active compounds onto novel metals (silver or gold) and then conjugating with specific biological recognition moieties to bring direct binding to the analyte for readout. In comparison to label-free SERS detection, stronger and simpler SERS signals could be produced by Raman-label compounds, further facilitating to achieve simple, multiple, and sensitive biological labeling. Due to the instability of SERS tags caused by environmental influence (such as pH, solvent and temperature), a variety of encapsulation approaches were developed to avoid the direct exposure of SERS tags to surrounding environments through covering the surface of nanopartilces with biocompatible materials, such as proteins,¹²³⁻¹²⁶ polymers¹²⁷ and silica^{108,109,128}. Although all these encapsulating species reduce the aggregation of SERS tags and facilitate their bio-functionalization, the silica-coated tags were shown to be superior due to their intrinsic properties: easy handling; reproducibility in preparation; impermeability to Raman molecules; remarkably immunity to changes in detection media; and ability to emit signals without influencing of bio-modification.¹⁰⁸ More importantly, silica encapsulation could also offer a well-defined core-shell structure, demonstrating specific and unambiguous composition of nanoprobles. With these advantages, considerable SERS-encoded probes have been applied for biological

targeting and imaging, including small molecules, DNA, proteins, cells, tissues and even tumors in vivo.^{107,109,127 105,123-125,129}

In Chapter 4, we synthesized a SERS probe conjugated with vancomycin affinity groups. For this SERS bio-tag, gold and silver core/shell nanoparticles are served as Raman enhancement substrate and protected by a layer of silica shell. Vancomycin molecules are modified on the surface of silica shell to form polyvalent ligand binding system. Upon the multivalent interaction between polyvalent vancomycin ligands and bacterial cell walls, the SERS bio-tag exhibits excellent labeling properties for Raman imaging of both vancomycin sensitive and resistant bacteria, which demonstrated that it is possible for this SERS probe to substitute fluorescent labeling in optical imaging of living bacteria.

1.4 Research topics and goals

Vancomycin, a kind of glycopeptide antibiotic, is often used to treat clinic infections caused by Gram-positive bacteria. Its mode of action involves the selective binding to the D-Ala-D-Ala termini of peptidoglycan in bacterial cell walls. The complexation of vancomycin and terminal peptides acts as a steric obstacle that inhibits the biosynthesis of peptidoglycan, which is an essential component of bacterial cell wall. The loss of peptidoglycan results in weakening the rigid cell walls and leaving bacteria susceptible to lysis because of changes in osmotic pressure. However, with the overuse of this antibiotic molecule, some drug resistant bacterial strains have emerged in recent years. These Gram-positive bacteria pathogens such as

vancomycin-resistant *enterococci* (VRE) mutate their terminal peptides from D-Ala-D-Ala to D-Ala-D-Lac, which shows three orders of magnitude decreasing in binding affinity with vancomycin antibiotic. Our research works mainly focus on vancomycin molecule, with the aims: (1) understand the detailed structure and activity of vancomycin towards cell wall peptide analogs; (2) design novel vancomycin derivatives as photodynamic antimicrobial agents; (3) image drug resistant bacteria based on vancomycin modified SERS probes.

In order to understand the driving force behind the associations of antibiotic and bacterial cell walls, Chapter 2 presented a systematic study to investigate the interactions between vancomycin and bacterial cell wall mimicking peptide ligands in the commonly employed buffer systems (phosphate and HEPES buffers) using single molecule fluorescence correlation spectroscopy (FCS) and molecular dynamics (MD) simulations. The FCS measurements revealed that the components of the buffer system take part in molecular recognition through binding to pocket of vancomycin, extremely disturbing the intrinsic stability of the complexes formed between vancomycin and low-affinity ligand, D-Ala-D-Lac. Therefore, surrounding environment should be one of most important considerations for the novel antibiotic designs. Exclusion of the interference from neighboring chemical or biological species could be a promising step towards increasing the efficacy of antibiotic drugs against bacterial pathogens.

The occurrence of VRE strains is due to the undesired peptide termini mutations, rendering drug ineffective in the fight against related infections. Its worldwide

prevalence has become a serious threat to public health. Therefore, it is necessary to develop novel antimicrobial agents against VRE bacterial infections. Chapter 3 described a simple and novel phototherapeutic reagent by conjugating porphyrin with two vancomycin moieties. This vancomycin-porphyrin conjugate exhibited an excellent photodynamic antibacterial activity against VRE pathogenic bacteria through strong and specific binding to bacterial surface. In addition, this divalent vancomycin-porphyrin also could be served as a useful fluorescent probe for noninvasive imaging study on living bacteria.

In Chapter 4, polyvalent vancomycin was used as affinity group for specifically targeting of Gram-positive bacteria, including vancomycin sensitive and resistant bacterial strains. A vancomycin modified SERS probe was synthesized through a rapid and straightforward method, in which Raman reporter labeled silver/gold core-shell nanoparticles were coated with silica protective shell and further modified with vancomycin. Detailed studies on living bacterial Raman imaging were performed to evaluate its possibility to be used for a substituent of fluorescent labels in bacterial optical imaging.

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Chapter 2

Molecular Interaction Between Glycopeptide Vancomycin and Bacterial Cell Wall Peptide Analogs

2.1 Introduction

The glycopeptide antibiotic vancomycin (Van) is often used as a last resort to effectively treat methicillin resistant *Staphylococcus aureus* (MRSA).¹⁻³ The mode of action of Van involves the selective binding of the antibiotic to the -D-Ala-D-Ala termini of the bacteria cell wall peptidoglycan which leads to an inhibition of cell growth and eventual cell death. With the frequent use of this antibiotic molecule, some drug resistant Gram positive bacterial pathogens such as vancomycin-resistant *enterococci* (VRE) have emerged, which are capable of assembling the peptidoglycan precursors and switching the termini -D-Ala-D-Ala to -D-Ala-D-Lac.^{4,5} This undesired microbial development results in a drastic drop in the binding affinity between Van and the resistant-bacteria and renders the drug ineffective in the fight against VRE-related infections, which have now become a major and worldwide public health threat. There is therefore a new urgency to understand the detailed structure and activity of vancomycin antibiotic derivatives with the aim of developing new drug candidates against evolving bacterial pathogens.^{4,5}

In order to design Van derivatives that exhibit improved antimicrobial activity against both Van-receptive and Van-resistant bacteria, it is necessary to understand the driving force behind the interaction between the antibiotic and bacteria cell wall mimicking peptide ligands. The binding affinities between Van and the ligands

N,N'-Diacetyl-L-Lys-D-Ala-D-Ala (Ac₂KDADA) and *N,N'*-Diacetyl-L-Lys-D-Ala-D-Lac (Ac₂KDADL) in buffer solutions have been previously investigated using several methods including UV-difference spectroscopy,^{6,7} fluorescence,⁸⁻¹⁰ affinity capillary electrophoresis,^{11,12} isothermal titration microcalorimetry,¹³ and nuclear magnetic resonance.^{14,15} The association between Van and cell wall analogs has also been studied in the gas-phase using mass spectrometry.¹⁶ There exists discrepancy between the various reported binding constant values which has been ascribed to different experimental techniques and analysis methods used.^{17,18} The degree of association between Van and Ac₂KDADA has also been shown to be affected by solvent properties such as pH, ionic strength and the type of solvents used, indicating, in general, the complexity of the intermolecular interaction.^{9,19} Despite extensive studies on the environmental effects on the binding between Van derivatives and bacterial cell wall peptide precursors, the exact nature of the inconsistency in the equilibrium binding constant values reported so far remains vague.^{17,18} In buffer solutions, detailed information on the effects of the various components that make up the buffer (*e.g.*, phosphate ion in phosphate buffer etc.) on the association between Van and peptide analogs need to be further explored since buffer solutions are known to interfere with the coupling between ligands and biological receptors and their effects should therefore be carefully characterized.^{20,21}

In this study, single molecule fluorescence correlation spectroscopy (FCS) is firstly used to investigate the association between Van and cell wall analogs terminating in -D-Ala-D-Ala and -D-Ala-D-Lac in the commonly employed vancomycin and

bacterial cell wall peptide precursors interaction buffer systems; phosphate and HEPES buffers. FCS is the technique based on the analysis of the diffusion of an emitting molecule as it diffuses in the excitation volume of a confocal microscope (Scheme 2.1). The difference in diffusion behavior between a freely diffusing emitting molecule and when it binds to a target allows the quantification of the corresponding binding constant between the two species. FCS is now recognized to be a very simple yet powerful technique to study ligand-receptor interaction in biomolecular recognition.²²⁻²⁴ Unlike UV-difference spectroscopy and conventional fluorescence techniques, one major advantage of FCS is that it does not require the utilization of an environment-sensitive chromophore that alters its photophysical properties (*e.g.*, emission quantum yield, absorption coefficient) upon complex formation between the labeled ligand and substrate. In addition, FCS examines relatively low concentrations of peptide and Van, hence avoiding complications arising from peptide aggregation and Van dimerization which commonly occur when high concentrations of peptide/antibiotics are used.^{11,12} Herein, the peptide sequences AcK(NH₂)DADA and AcK(NH₂)DADL are labeled with ATTO 655 which is a photostable and commonly used near-infrared fluorescent (NIR) dye in FCS studies.²⁵ The molecular interaction between the fluorescent labeled/non-labeled peptide sequences and Van and the influence of phosphate and HEPES buffers on the intrinsic binding between the two moieties are systematically investigated using both single molecule FCS and molecular dynamics (MD) simulations.

Scheme 2.1. Schematic representation of the FCS study of the molecular interaction between Van and dye-labeled bacterial cell wall peptide analogs. A single complex formed by the association of the two moieties diffuses across the ellipsoidal excitation/observation volume.

2.2 Experimental Sections

2.2.1 Materials

Chemical reagents and solvents were used as received from Sigma-Aldrich unless otherwise stated. ATTO 655 NHS-ester, peptide precursors Ac₂KDADA and Ac₂KDADL, and 1 M HEPES buffer solution (pH 7.5) were purchased from ATTO-TECH GmbH (Germany), Bachem (Germany) and 1st BASE (Singapore). Vancomycin (Van, Sigma-Aldrich) was purified using a reversed-phase high performance liquid chromatography equipment (RP-HPLC, Shimadzu). For analytical RP-HPLC, the measurement was conducted with a Alltima C-18 column (250 × 3.0 mm) at a flow rate of 1.0 mL/min and semi-preparative RP-HPLC was performed on a C-18 column (250 × 10 mm) at a flow rate of 3 mL/min. Absorption and fluorescence

spectra were recorded using a 5-mm path quartz cell on a Beckman Coulter DU800 spectrometer and a Cary Eclipse (Varian) fluorescence spectrometer, respectively. Millipore-purified (Milli-Q) water was used in all experiments.

2.2.2 Preparation of peptide ligands

The peptide precursors (including AcK(NH₂)DADA and AcK(NH₂)DADL) were synthesized by solid-phase peptide synthesis using standard Fmoc chemistry. Briefly, 2-chlorotrityl chloride resin (200 mg, 1.0-1.5 mmol/g loading level) was placed into a reaction vessel of a peptide synthesizer and washed with DCM three times. The first amino acid was coupled to the resin under DIPEA (174.2 μ L, 1 mmol) in DCM, shaking for 30 min. After washing with MeOH and DMF, deprotection of Fmoc was conducted with 20% piperidine in DMF. The resin was then washed with DMF three times and subsequently subjected to the following series of coupling-deprotection cycles: (i) coupling with amino acid (0.4 mmol), HOBt (108.1 mg, 0.8 mmol), TBTU (256 mg, 0.8 mmol) and DIPEA (139.3 μ L, 0.8 mmol), shaking for 2 h, and (ii) Fmoc deprotection was carried out as stated above following three times DMF wash. After three cycles, the existed peptide sequence was coupled with acetic anhydride (1.2 mL, *v/v* 30%), DIPEA (1 mL, *v/v* 50%) and DMF (0.4 mL, *v/v* 20%) with shaking for overnight. The product was cleaved from the solid support by treatment with TFA/triisopropylsilane/anisole (2:1:1) and collected as white solids.

2.2.3 ATTO 655 labeled peptide ligands

After obtaining the peptide ligands, Atto655-NHS-ester (1 mg, 1 μ M) was labeled onto the peptides (1.4 mg, 4 μ M) by reacting for 4 h in anhydrous DMF and TEA under nitrogen. The products were purified by RP-HPLC and characterized using electrospray mass spectrometry. For the fluorescent-labeled peptides, the peaks at m/z 840.35 and 840.96 correspond to M^+ of AcK(ATTO655)DADA and AcK(ATTO655)DADL, respectively.

Figure 2.2. Structures and LC-Mass spectra data of the ATTO 655-labeled bacterial cell wall peptide analogs AcK(ATTO655)DADA and AcK(ATTO655)DADL.

For the fluorescence correlation spectroscopy measurements, the non-labeled peptide precursors, ATTO 655-labeled peptide ligands and vancomycin were dissolved in different solvents (neat water, phosphate buffer and HEPES buffer) to obtain the stock solutions. The concentration of the stock solution of ATTO 655-labeled peptide ligand was determined using the Beer-Lambert law. Solutions used in the FCS experiments were freshly prepared from stock solutions by serial dilution. For the FCS assay, the samples were prepared by mixing the dye-labeled peptide and Van in either neat water, phosphate buffer or HEPES buffer. In the case of the FCS competitive binding assay, the dye-labeled peptide, Van and

Ac₂KDADA/Ac₂KDADL were sequentially added into either neat water or buffer solutions. In all measurements, the final concentrations of dye-labeled peptide ligand and Ac₂KDADA/Ac₂KDADL are 1.5 nM and 5 mM, respectively, and the concentration of Van is between 0 and 50 μ M.

2.2.4 Fluorescence correlation spectroscopy (FCS)

FCS measurements were performed using a time-resolved confocal microscope (MicroTime 200, PicoQuant). The excitation source used is a 635 nm pulsed diode laser (LDH-P-C-635B, PicoQuant) with a repetition rate of 20 MHz. The excitation light passed through an excitation filter (Z636/10, Chroma) and directed *via* a dichroic mirror (Z638rpc, Chroma) into an inverted microscope (IX71, Olympus) before being focused onto the sample through a water objective lens (60 $\times$, N.A. 1.2, Olympus). The correction collar of the water immersion objective was optimized in order to obtain the highest signal-to-noise ratio and the fluorescence was subsequently collected by the same objective lens. The fluorescence was then passed through the dichroic mirror, an emission filter (HQ685/70, Chroma) and refocused through a pinhole (50 μ m in diameter) before being detected by an avalanche photodiode (SPCM-AQR-15, Perkin-Elmer). The excitation intensity of the laser beam used was 5.8 kW/cm².

The lateral radius (ω_0) and axial 1/e²-radius (z_0) of the confocal volume were determined by measuring the autocorrelation function of Atto655-COOH in water. The triplet state formation quantum yield of Atto655-COOH is negligible and the

fluorescence intensity fluctuations arise from the translational diffusion of the dye.²⁵⁻²⁷

Assuming that the confocal volume can be approximated by a 3-dimensional Gaussian function, ω_0 and z_0 were obtained by fitting the autocorrelation function ($G(t)$) of ATTO655-COOH in water using the following equation:

$$G(t) = G_0 \left(1 + \frac{t}{\tau} \right)^{-1} \left(1 + \frac{t}{\kappa^2 \tau} \right)^{-\frac{1}{2}} \quad (1)$$

The eccentricity of the confocal volume is defined by $\kappa = z_0/\omega_0$, and the diffusion time (τ) is related to ω_0 and the diffusion coefficient D via

$$\tau = \frac{\omega_0^2}{4D} \quad (2)$$

The diffusion coefficient of ATTO655-COOH in water at 23 °C is previously reported to be $D = 404 \mu\text{m}^2/\text{s}$.²⁵⁻²⁷ The number of fluorescent molecules (N) is obtained from $G(t)$ when $t = 0$ (*i.e.*, $N = G_0^{-1}$).

The binding constant of AcK(ATTO655)DADA was determined by measuring the FCS autocorrelation functions of the dye-labeled peptide ligand in varying amounts of Van. Basically, a dilute solution of AcK(ATTO655)DADA in either neat water or buffer solutions (concentration of 1.5 nM) was incubated with an appropriate amount of Van (between 0 to 50 μM) at ambient conditions before the FCS measurements were conducted. The experiment was repeated at least 3 times for each Van concentration and the results were averaged.

The binding equilibrium between Van (V) and AcK(ATTO655)DADA (L) is described by the reaction

where LV is the AcK(ATTO655)DADA:Van complex and the corresponding association constant (k_{al}) and dissociation constant (k_{dl}) are related by:

$$k_{al} = \frac{1}{k_{dl}} = \frac{[LV]}{[L][V]} \quad (4)$$

When an intermediate concentration of Van is incubated with AcK(ATTO655)DADA, the autocorrelation function of the sample solution measured is best fitted to a two-component model that takes into account the diffusion times of both the free labeled peptide ligand ($\tau_L = 98.8 \mu\text{s}$) and the labeled bound complex ($\tau_{LV} = 128 \mu\text{s}$):

$$G(t) = N^{-1} \left[(1-Y) \left(1 + \frac{t}{\tau_L} \right)^{-1} \left(1 + \frac{t}{\kappa^2 \tau_L} \right)^{-\frac{1}{2}} + Y \left(1 + \frac{t}{\tau_{LV}} \right)^{-1} \left(1 + \frac{t}{\kappa^2 \tau_{LV}} \right)^{-\frac{1}{2}} \right] \quad (5)$$

with the relative proportion of LV given by

$$Y = \frac{[LV]}{[L] + [LV]} \quad (6)$$

The dissociation constant k_{dl} is obtained by fitting the graph of Y against $[V]$ using the following equation:²⁸

$$Y = [L]_0^{-1} \left[\left(\frac{k_{dl} + [L]_0 + [V]_0}{2} \right) - \sqrt{\left(\frac{k_{dl} + [L]_0 + [V]_0}{2} \right)^2 - [L]_0 [V]_0} \right] \quad (7)$$

where $[L]_0$ and $[V]_0$ are the initial concentrations of L and V , respectively.

The binding constants between Van and the unlabeled Ac₂KDADA and Ac₂KDADL peptides (I) are determined using the method of competitive binding. In this case, Van is added to a mixture containing 1.5 nM AcK(ATTO655)DADA and 5 mM peptide Ac₂KDADL. The association constant for the formation of

AcK(ATTO655)DADA:Van complex is given by equation (4) while the association (k_{a2}) and dissociation (k_{d2}) constants for binding between Van (V) and I (i.e., $I + V \rightleftharpoons IV$) are:

$$k_{a2} = \frac{1}{k_{d2}} = \frac{[IV]}{[I][V]} \quad (8)$$

The relationship between the initial concentration of Van ($[V]_0$) and the binding proportion (Y) of AcK(ATTO655)DADA to Van, in competition with the unlabeled peptide I , is expressed as:²⁹

$$[V]_0 = \frac{Y k_{d1}}{1 - Y} + [L]_0 Y + \frac{[I]_0 Y k_{d1}}{k_{d2} (1 - Y) + Y k_{d1}} \quad (9)$$

where $[L]_0$ and $[I]_0$ are the initial concentrations of AcK(ATTO655)DADA and the unlabeled peptide I , respectively, and Y , k_{d1} and k_{d2} are defined in equations (7), (4) and (8), respectively.

2.2.5 Molecular dynamics (MD) simulation

Each molecule was first optimized at the AM1 level using the Gaussian 03 package and the minimized structure was used to calculate the B3LYP/6-31G* electrostatic potential (ESP). The atomic charges used for molecular mechanics calculations were derived from the ESP by using the RESP program implemented in the AMBER 9.0 package.

For the ligands (Ac₂KDADA, Ac₂KDADL, HEPES, HPO₄²⁻ and H₂PO₄⁻) studied here, the binding orientations were first estimated using AutoDock 4.0. The preliminary docking study shows that the vancomycin carboxylate binding site is the

site for docking to either sulphate or phosphate. We thus used this site to generate the receptor site and the energetic grids for the docking calculations. Flexible docking was performed in which single bonds outside the rings were set free to rotate. During the docking process, conformational search was performed using the Solis and Wets local search method, and the Lamarckian genetic algorithm was applied to find minimum energy structure of the ligand–receptor complexes.

The docking structures obtained were then minimized using 1000 steps of Steepest Descent followed by another 3000 steps of Conjugate Gradient. Molecular dynamics simulations were carried out using the SANDER module of the AMBER 9.0 program. For each binding complex obtained from AutoDock, about 2000 TIP3P water molecules with 10 Å buffer were added around the complex. K⁺ counterions were added to maintain the neutrality of the system. The simulations were carried out at 300 K with a time step of 1.0 fs. The non-bonded cutoff was set to 10.0 Å and SHAKE algorithm was applied for all the bonds involving hydrogen atoms. After minimization of 4000 steps and equilibration for 100 ps, complex conformations were collected every 0.5 ps for the following 10 ns simulation. Finally, 1000 snapshots were collected from the region with stable fluctuation for post-processing analysis and free energy calculations.

For each snapshot collected during the MD simulation, both interaction energies (ΔE^{vdw} , ΔE^{elec}) and the electrostatic contribution ($\Delta\Delta G^{PB}$) to the solvation energy were calculated with the PBSA program of AMBER 9.0. The non-polar part of the solvation energy ($\Delta\Delta G^{SA}$) is estimated using the simple empirical relation: $\Delta\Delta G^{SA} =$

$cA + b$, where A is the solvent-accessible surface area that is estimated using Sanner's algorithm implemented in the MSMS program with a solvent probe radius of 1.4 Å and the PARSE atomic radii parameters. c and b are empirical constants and are set to 0.00542 and 0.92 kcal/mol, respectively. The energy terms obtained with MM-PBSA approach were then averaged over 100 time frames. The normal mode calculation to estimate the entropy contribution was performed by using Nmode module in the AMBER 9.0 program.

2.3 Results and discussions

2.3.1 Direct molecular binding between AcK(ATTO655)DADA and Van in neat water

The peptide precursors were first modified by ATTO 655 NHS and further purified by RP-HPLC. The diffusion coefficient of ATTO 655 remains invariant in the different solvents used in this study indicating the insignificant effect of viscosity. In addition, the change in emission quantum yield when the labeled peptide ligand is bound to the antibiotic is small ($< 10\%$) and is not considered in further analysis.²⁸ The FCS autocorrelation functions of the fluorescent labeled AcK(ATTO655)DADA and AcK(ATTO655)DADA:Van complex in neat water yielded diffusion coefficients of $D = 310 \pm 2 \mu\text{m}^2 \text{s}^{-1}$ and $240 \pm 2 \mu\text{m}^2 \text{s}^{-1}$, respectively. As expected, in the presence of Van, the labeled peptide ligand bound to Van diffuses more slowly across the observed volume. Figure 2.3 shows the normalized autocorrelation functions of freely diffusing ATTO 655 dye, AcK(ATTO655)DADA and AcK(ATTO655)DADA in the

presence of 50 μM of Van in neat water. The autocorrelation function of AcK(ATTO655)DADA is well fitted to equation (1) which yields a diffusion time of $\tau = 98.8 \mu\text{s}$ and a corresponding D value, obtained from equation (2), of $310 \mu\text{m}^2/\text{s}$. In the presence of Van, a slower fluorescence intensity fluctuation is observed when the labeled peptide ligand bound to Van diffuses more slowly across the observed volume. At high Van concentrations (*i.e.*, 50 μM), the autocorrelation function is best described using equation (1) with $\tau = 128 \mu\text{s}$. This suggests an insignificant amount of free labeled peptide ligand present in the 50 μM Van solution and the diffusion time of 128 μs is ascribed to the diffusion of AcK(ATTO655)DADA:Van complex across the observed volume. From equation (2), a value of $D = 240 \mu\text{m}^2/\text{s}$ is calculated for the bound complex. Figure 2.4 shows the graph of the binding percentage of the ligand (L) to Van (V) (*i.e.*, $Y = [LV]/([L] + [V])$), against various concentrations of Van $[V]$, and clearly indicates an increase in binding upon increasing $[V]$. Based on this fluorescence binding assay, a dissociation constant of $k_d = 1.1 \pm 0.1 \mu\text{M}$ is obtained for AcK(ATTO655)DADA in neat water.

Figure 2.3. Normalized autocorrelation functions of ATTO 655 dye ($\square$), AcK(ATTO655)DADA ($\circ$) and AcK(ATTO655)DADA in the presence of 50 μM of Van (Δ) in neat water.

Figure 2.4. The Y versus [Van] plots for AcK(ATTO655)DADA in neat water ($\circ$) and AcK(ATTO655)DADA in the presence of 5 mM Ac₂KDADL in neat water ($\square$), 5 mM phosphate buffer (Δ) and 10 mM phosphate buffer ($\diamond$).

2.3.2 Competitive binding to Van between AcK(ATTO655)DADA and Ac₂KDADL in neat water

The binding constant of labeled AcK(ATTO655)DADL to Van cannot be directly determined using FCS since the autocorrelation function in neat water does not vary significantly when Van is added into the solution. This demonstrates that the binding between Van and the low-affinity ligand is negligible for the concentrations of peptide

(1.5 nM) and Van (between 0 and 50 μ M) used in the FCS experiment. Larger amounts of Van are avoided due to possible dimerization of the antibiotics at high concentrations.^{11,12} In order to precisely investigate the weak Van-binding affinity of the peptide with the D-Ala-D-Lac residue, a FCS-based competitive binding assay is used in which the non-labeled Ac₂KDADL ligand compete with AcK(ATTO655)DADA for binding to Van (see Experimental sections). The Y vs. $[V]$ plot for AcK(ATTO655)DADL in the presence of 5 mM of Ac₂KDADL is shown in Figure 2.4, and the k_d value in neat water for Ac₂KDADL is determined to be 260 ± 6 μ M. The competitive binding assay is also used to determine the Van-binding affinity of Ac₂KDADA which is found to be similar to that of AcK(ATTO655)DADA (Figure 2.5), hence suggesting that the appended fluorophore does not affect the binding equilibrium. We note that the Van-binding affinity of the peptide ligand increases in the order of D-Ala-D-Lac < D-Ala-D-Ala. The loss of a hydrogen bond and a concomitant gain of an oxygen-oxygen lone pair repulsion between Ac₂KDADL and Van are, in part, responsible for the significant reduction in the degree of binding between the two moieties when compared to the peptide terminating in D-Ala-D-Ala (see Figure 2.6).^{6,30}

Figure 2.5. The Y versus $[Van]$ plots for AcK(ATTO655)DADA in the presence of 5 μM Ac₂KDADA in neat water. The k_d value for the binding between Van and Ac₂KDADA, obtained by fitting the above curve to equation (9), is $0.8 \pm 0.1 \mu\text{M}$.

2.3.3 Binding interactions in Phosphate buffer

The effects of buffers on the binding between Van and the various peptides are discussed next. When the solvent is changed from neat water to a 10 mM phosphate buffer (consisting of 6.8 mM HPO_4^{2-} and 3.2 mM H_2PO_4^- , pH = 7.3, ionic strength μ = 24 mM), the Van-binding affinity of AcK(ATTO655)DADA (*i.e.*, $k_d = 1.3 \mu\text{M}$) does not vary significantly from the one observed in neat water, and the k_d determined by FCS is in agreement with previously reported values in phosphate buffers.⁸ This suggests that the high affinity of AcK(ATTO655)DADA to Van is not influenced by the phosphate buffer. Figure 2.4 shows the Y vs. $[V]$ plots of AcK(ATTO655)DADA in the presence of 5 mM Ac₂KDADL in 5 mM phosphate buffer (consisting 3.4 mM

HPO_4^{2-} and 1.6 mM H_2PO_4^- , pH = 7.3, μ = 12 mM) and 10 mM phosphate buffer. It is observed that as the concentration of phosphate ions increases, a smaller initial concentration of Van is needed to achieve a large (relative) proportion of AcK(ATTO655)DADA:Van complex (*i.e.*, Y). This means that the binding strength between Van and Ac₂KDADL significantly decreases when the phosphate buffer concentration is increased from 0 to 10 mM. The k_d value of Ac₂KDADL, obtained from Figure 2.4, increases from 260 μM in neat water to $1040 \pm 20 \mu\text{M}$ and $2140 \pm 40 \mu\text{M}$ in 5 mM and 10 mM phosphate buffers, respectively. When the phosphate ion concentration is further increased by utilizing a 20 mM phosphate buffer (consisting of 13.7 mM HPO_4^{2-} and 6.3 mM H_2PO_4^- , pH = 7.3, ionic strength μ = 47 mM), the stability of the Ac₂KDADL:Van complex yields a k_d ($= 1850 \pm 50 \mu\text{M}$) value close to that measured in the 10 mM phosphate buffer. It is worth noting that the k_d values, determined *via* FCS, for the 10 mM and 20 mM phosphate buffers fall in the range previously reported for Ac₂KDADL in phosphate buffers (*i.e.*, *ca.* 1800 μM to 3000 μM for buffer concentrations of 20 mM to 0.2 M).^{8,11,12,17}

Figure 2.6. The binding interaction between the glycopeptide antibiotic vancomycin and Ac₂KDADA (a), Ac₂KDADL (b), HEPES (c) and HPO₄²⁻ (d). Hydrogen bonds are indicated by dotted lines.

2.3.4 Binding interactions in HEPES buffer

Similar behavior is also observed when the FCS measurements are conducted in HEPES buffer. In this case, $k_d = 1.1 \pm 0.1 \mu\text{M}$ is obtained for AcK(Atto655)DADA in 10 mM HEPES, 6 mM NaCl buffer (pH = 7.3, $\mu = 10 \text{ mM}$). Perkins and Nieto have shown that the stability of the Ac₂KDADA:Van complex remains relatively unchanged in the pH range from 3 to 8 and at low ionic strengths as employed in this study.¹⁹ Our results, therefore, suggest that the binding of the peptide terminating in D-Ala-D-Ala to Van is not affected by the buffer solution due to its high binding affinity. On the other hand, the association between Ac₂KDADL and Van is weakened

as the concentration of HEPES increases (Figure 2.7). From the ligand competitive assay, k_d values for Ac₂KDADL in 5 mM HEPES, 6 mM NaCl buffer (pH = 7.3, μ = 8 mM) and 10 mM HEPES, 6 mM NaCl buffer are determined to be 677 ± 17 μ M and 2870 ± 189 μ M, respectively. We note an *ca.* 8- to 11- times enhancement in k_d value on going from neat water solvent to 10 mM phosphate or 10 mM HEPES, 6 mM NaCl buffer solutions, further confirming that the components constituting the buffers (*i.e.*, phosphate ions and HEPES) affect the molecular interaction between Van subunits and bacterial cell wall peptide analogs.

Figure 2.7. The Y versus [Van] plots for AcK(ATTO655)DADA in the presence of 5 mM Ac₂KDADL in 5 mM HEPES, 6 mM NaCl buffer (○) and 10 mM HEPES, 6 mM NaCl buffer (Δ).

2.3.5 Ionic strength effect for binding constants

To determine if ionic strength plays a role in changing the Van-binding constant of Ac₂KDADL in phosphate buffer, the binding of Ac₂KDADL to Van in 5 mM phosphate, 12 mM NaCl buffer (pH = 7.3, μ = 24 mM) is determined using FCS and a

k_d value of $1170 \pm 70 \mu\text{M}$ is obtained which is close to the k_d value measured in 5 mM phosphate buffer (*i.e.*, in the absence of NaCl). Similarly, the k_d ($= 3000 \pm 80 \mu\text{M}$) value determined from the FCS experiment in 10 mM HEPES buffer ($\text{pH} = 7.3$, $\mu = 4$ mM) is close to that obtained in the 10 mM HEPES, 6 mM NaCl buffer. Therefore, ionic strengths used in this study do not significantly alter the binding/dissociation constant. The values of k_d and the corresponding change in free energy of binding ($\Delta G = -RT \ln k_d^{-1}$) for the peptides in different solvents are presented in Table 2.1.

Table 2.1. FCS determined k_d and ΔG for interaction between Van and AcK(ATTO655)DADA and Ac₂KDADL.

Ligand	Solvent	k_d (μM)	ΔG (kcal/mol)
AcK(ATTO655)DADA	neat water	1.1 ± 0.1	-8.1
	10 mM phosphate buffer	1.3 ± 0.1	-8.0
	10 mM HEPES, 6 mM NaCl buffer	1.1 ± 0.1	-8.1
Ac ₂ KDADL	neat water	260 ± 6	-4.9
	5 mM phosphate buffer	1040 ± 20	-4.0
	10 mM phosphate buffer	2140 ± 40	-3.6
	5 mM HEPES, 6 mM NaCl buffer	677 ± 17	-4.3
	10 mM HEPES, 6 mM NaCl buffer	2870 ± 189	-3.4

$$^a \Delta G = -RT \ln k_d^{-1}$$

2.3.6 MD simulations for binding complexes

To further assess the stability of the complexes formed between Van and the various affinity ligands (*i.e.*, peptide precursors Ac₂KDADA and Ac₂KDADL, buffer ions H₂PO₄⁻ and HPO₄²⁻, and HEPES), MD simulation in an explicit water environment is performed and the values of the MD computed change in free energy of binding ΔG_{md} are given in Table 2.2. The qualitative trend in ΔG_{md} indicates a distribution of hydrogen bonding strengths in the binding complexes of Van with Ac₂KDADA, Ac₂KDADL, H₂PO₄⁻, HPO₄²⁻, and HEPES. According to the MD simulation, the carboxylate headgroups of Ac₂KDADA and Ac₂KDADL, sulfate group of HEPES, and phosphate group of HPO₄²⁻ are oriented toward the Van carboxylate binding site such that a minimal set of hydrogen bonding exists between the peptide/ligand and the antibiotic amide NH groups found in residues 2, 3, and 4 (hydrogen bonds labeled 1, 2 and 3 in Figure 2.6). We note that the value of ΔG_{md} is reduced from -14.7 kcal mol⁻¹ to -6.0 kcal mol⁻¹ on going from Ac₂KDADA to Ac₂KDADL. This is due to the missing central hydrogen bond (*i.e.*, hydrogen bond labeled 4 in Figure 2.6(a)) when the NH is substituted for an O in Ac₂KDADL, resulting in the weakening of the hydrogen bond between the peptide and the amide NH found in both residue 4 (hydrogen bond labeled 3 in Figure 2.6(b)) and residue 7 (hydrogen bond labeled 5 in Figure 2.6(b)). The MD simulation also reveals that the binding of Van to the hydrogen phosphate ion HPO₄²⁻ ($\Delta G_{\text{md}} = -7.4$ kcal/mol) appears to be slightly stronger than the binding to Ac₂KDADL but much weaker than the association between Ac₂KDADA and Van. Apart from hydrogen bonding with the

amide NH groups of residues 2, 3, and 4 of Van, the complex formed between HPO_4^{2-} and Van is further stabilized by additional hydrogen bonds involving the amide NH group in residue 1 and the carbonyl group in residue 4 (hydrogen bonds labeled 6 and 7 in Figure 2.6(d)). On the other hand, dihydrogen phosphate ion H_2PO_4^- appears to be bound only very weakly to the binding pocket of the antibiotic (see Figure 2.8). Both Ac_2KDADL and HEPES undergo complexation with Van by means of three hydrogen bonds, however, the flexible framework of HEPES molecule under the influence of solvent molecules impairs its Van-binding affinity and leads to a lower free energy of binding ΔG_{md} (-3.1 kcal/mol).

The MD calculated binding energies and entropies from normal mode analysis are presented in Table 2.2 and the percent occupancy of hydrogen bonds with 3.0 Å and 120° cutoffs for the distance and angle, respectively, are listed in Table 2.3. Figure 2.8 shows the last snapshot of the binding complex formed between Van and the ligands Ac_2KDADA , Ac_2KDADL , HEPES and HPO_4^{2-} . As seen, the carboxylate headgroups of Ac_2KDADA and Ac_2KDADL , sulfate group of HEPES and phosphate group of HPO_4^{2-} are oriented toward the Van carboxylate binding site and held in place through a network of hydrogen bonds. Compared to Ac_2KDADA , the extent of Van binding with Ac_2KDADL is reduced significantly due to the complete loss of hydrogen bonding interaction with the carbonyl in residue 4 of Van and the concomitant weakening of hydrogen bond with the amide NH in residues 4 and 7 of Van (see Table 2.3 and Figure 2.8). Similarly, Ac_2KDADL and HEPES are bound to the Van carboxylate binding pocket by means of three hydrogen bonds. However, the flexible

framework of HEPES molecule under the influence of solvent molecules could impair the binding affinity further. Another interesting feature is the binding of Van with hydrogen phosphate HPO_4^{2-} . MD simulation result indicated that this small anion is located in a similar position and orientation as Ac_2KDADA carboxylate group and stabilized by a network of hydrogen bonds including the ones with the amide NH group in residue 1 and carbonyl group in residue 4. Figure 2.9 shows the root-mean-square deviation (RMSD) fluctuations of the ligands and Van against the simulation time. It can be seen clearly that RMSD of the dihydrogen phosphate H_2PO_4^- fluctuates dramatically and leaves the original binding site around 9 ns of simulation, indicating that H_2PO_4^- cannot be stabilized within the carboxylate binding site of Van. On the other hand, HPO_4^{2-} remains stable within the binding pocket of Van even for simulation time up to 50 ns (data not shown).

Table 2.2. Binding free energies (kcal/mol) of Van with the various ligands.

ligand	ΔE^{vdw}	ΔE^{elec}	$\Delta E^{inter \dagger}$	$\Delta \Delta G^{PB}$	$\Delta \Delta G^{SA}$	$\Delta G^{MMPBSA \ddagger}$	$T\Delta S$	ΔG_{md}^{ξ}
Ac_2KDADA	-25.37	-135.14	-160.51	129.71	-3.94	-34.73	-20.03	-14.70
Ac_2KDADL	-22.14	-119.23	-141.37	118.09	-3.90	-27.19	-20.19	-7.00
HEPES	-19.72	-72.59	-92.29	75.89	-3.19	-19.59	-16.45	-3.14
HPO_4^{2-}	-3.35	-282.17	-285.52	266.99	-2.21	-20.73	-13.35	-7.38

$$\dagger \Delta E^{inter} = \Delta E^{vdw} + \Delta E^{elec}$$

$$\ddagger \Delta G^{MMPBSA} = \Delta E^{inter} + \Delta \Delta G^{PB} + \Delta \Delta G^{SA}$$

$$\xi \Delta G_{md} = \Delta G^{MMPBSA} - T\Delta S$$

Figure 2.8. The final snapshot of the 10-ns MD simulation of vancomycin binding complex with Ac₂KDADA (A), Ac₂KDADL (B), HEPES (C) and HPO₄²⁻ (D).

Table 2.3. Hydrogen bonds percent occupancies with 3.0 Å and 120° cutoffs for the distance and angle, respectively.

	1		2		3		4	5
Ac ₂ KDADA	77.34	14.77	53.92	9.67	62.98	7.68	14.16	65.57
Ac ₂ KDADL	45.27	41.76	42.15	19.74	20.49	11.95	0	14.82
HEPES	35.23	15.62	50.50	10.49	31.83	5.78	0	0
HPO ₄ ²⁻	83.93	0.62	91.01	1.67	37.55	22.43	39.24	0

Figure 2.9. Root-mean-square deviations RMSD (Å) of Ac₂KDADA, Ac₂KDADL, HEPES, HPO₄²⁻, H₂PO₄⁻ (red) and Van (black) for 10 ns MD simulations.

2.3.7 Binding complexes between HPO₄²⁻ or HEPES and Van

In our FCS measurement, the reduction in the binding strength between Van and Ac₂KDADL, resulting from a change of solvent from neat water to buffer solution, may arise from effects associated with the HPO₄²⁻-Van and HEPES-Van interaction. Aoki *et al.* have reported the crystal structures of complexes formed between Van and peptides terminating with the sequence D-Ala-D-Lac and have shown that hydrogen bonding interaction between phosphate ions and Van is non-negligible.³¹ In neat water, Ac₂KDADL is able to replace solvent water found in the antibiotic binding pocket

after both desolvation and conformational rearrangement of the binding site have taken place.³² On the other hand, expulsion of HPO_4^{2-} and HEPES from the binding pocket is relatively more difficult due to their stronger interactions with Van. Based on recent crystal structure studies,^{31,33} a simple model is proposed whereby a water-mediated hydrogen bond interaction between the amide nitrogen of the Lys residue in Ac₂KDADL and the oxygen of the terminal carboxylate group of Van is necessary to achieve favorable peptide orientation and energy to remove and replace HPO_4^{2-} and HEPES from the binding site. This results in a loose Ac₂KDADL:Van complex in buffer solution as compared to neat water. On average, the number of Ac₂KDADL:Van complex decreases while the number of water, HPO_4^{2-} and HEPES filled Van available for binding to Ac(Atto655)KDADA increases as the buffer concentration is raised until an equilibrium point is reached. Ac₂KDADA binds strongly to Van and can therefore easily replace HPO_4^{2-} and HEPES from the binding pocket without significant variation in the stability of the complex formed. Other considerations such as cooperativity³⁴ between the various factors influencing binding in the presence of HPO_4^{2-} and HEPES may be important but the exact nature of their roles is not clear at the present moment.

2.4 Conclusions

In this Chapter, we have presented a systematic study to investigate the molecular interactions of glycopeptide antibiotic vancomycin with different affinity ligands including Van-sensitive and Van-resistant bacterial cell wall peptide analogs and buffer ions using both competitive FCS measurement and MD simulation. The FCS measurement clearly indicates a higher Van-binding affinity for the drug-sensitive bacteria cell wall precursor Ac₂KDADA as compared to the drug-resistant bacterial cell wall peptide analog Ac₂KDADL. The competitive FCS and MD simulation studies also demonstrate the profound effects of phosphate and HEPES buffers on the stability of the complexes formed between Van and the low-affinity Ac₂KDADL. The weak association between the buffer components and the binding pocket of Van has several important implications. In particular, the effects of phosphate and HEPES buffers on the binding between Van and antibiotic-resistant peptide analogs are significant and should not be ignored. Furthermore, there may exist components in the Van-resistant bacteria cell wall and its surrounding environment that are capable of binding to the antibiotic molecules leading to a perturbation and possible reduction in the drug's ability to directly associate and kill bacteria. Therefore, the design of Van derivatives that bind specifically to the termini D-Ala-D-Lac of the peptidoglycan without interference from surrounding chemical or biological species is a promising step towards increasing the efficacy of antibiotic drugs against bacterial pathogens and obtaining a more thorough understanding of the actual host-guest reaction mechanism of antibiotics.³⁵

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Chapter 3

Multifunctional Divalent Vancomycin: The Fluorescent Imaging and Photodynamic Antimicrobial Properties for Drug Resistant Bacteria

3.1 Introduction

In recent decades, there has been an increasing in world-wide threat to public health due to vancomycin-resistant *enterococci* (VRE). These bacteria having a highly resistant to vancomycin reprogram of peptidoglycan terminus from D-Ala-D-Ala to D-Ala-D-Lac. This mutation leads to a nearly 1000-fold drop in binding affinity to Van molecule.^{1,2} Based on extensive studies done by Griffin,³ Nicolaou,⁴ Williams⁵ and Whitesides *et al.*,^{6,7} covalently linked dimers and oligomers of Van could serve as promising approaches to effectively against drug-resistant pathogenic bacteria. These Van derivatives circumvent the low affinities binding to D-Ala-D-Lac peptide precursors *via* the polyvalent/multivalent interactions with resistant bacterial cell wall, exhibiting enhanced antimicrobial potency.⁸⁻¹⁰ However, recent research works by Williams *et al.* also verified that increased binding affinity may not always lead to substantial activities with effective minimum inhibitory concentration (MIC) against VRE organisms.^{5,11,12} Thus, there is still an urgency to search for alternative treatment strategies against bacterial infections caused by VRE strains.

Photodynamic antimicrobial chemotherapy (PACT) has received considerable attention to serve as a promising alternative approach for microbiological control.¹³ In

this strategy, reactive oxygen species (ROS, *e.g.* singlet oxygen ($^1\text{O}_2$)) are generated by photosensitizers (PS) upon the irradiation with a suitable wavelength light. These cytotoxic ROS lead to destroyed cell walls and membranes and finally cell death.^{14,15} So far, PACT has been extensively applied for the treatment of variety of pathogen infections caused by both Gram-positive and Gram-negative bacteria.¹³⁻¹⁵ However, a substantial concern for PACT treatments is to control the collateral damage of ROS to host tissue. The utilization of affinity ligands serves as a promising strategy to specifically target photosensitizers to areas of bacterial infections, thus minimizing side effects and further improving the efficiency of PACT in clinics. Several affinity ligands based on antibodies,¹⁶ protein cage,¹⁷ polypeptide,¹⁸ nanoparticles,¹⁹ and bacteriophage²⁰ have been reported to successfully direct lethal photosensitizers to antibiotic-resistant bacteria. However, the development of simpler and economical novel targeting molecules capable of specifically directing photosensitizers to drug resistant bacteria remains necessary and is of great significance since most of current approaches are complicated, require tedious manipulation and may suffer from difficulty in synthesis, self-aggregation or possible immunogenicity.¹⁶⁻²⁰

In this study, Van antibiotic was employed as the affinity ligand, and porphyrin, a commonly used photosensitizer due to its clinical significance in both PACT^{13,21,22} and noninvasive fluorescent imaging of living cells *in vitro* and *in vivo*,²³⁻²⁶ was chosen as the bridging moiety to generate Van conjugated multivalent/polyvalent dimeric system (Scheme 3.1). The divalent Van-porphyrin conjugate possesses several unique advantages including (i) ease of preparation, (ii) the rigid structure of the

porphyrin linker supplies entropically enhanced binding and steric hindrance necessary for multivalent/polyvalent interactions between the disubstituted Van and VRE strains,⁶⁻¹⁰ (iii) selective adhesion of divalent Van to bacterial surfaces leading to enhanced photodynamic inactivation of Van-sensitive and VRE strains which are more potent than the MICs of Van itself, and (iv) the divalent Van serves as a promising fluorescent probe to label and monitor bacterial strains in a highly effective manner.

Scheme 3.1. Interaction of divalent vancomycin and Gram-positive bacterial cell wall.

3.2 Experimental sections

3.2.1 General

Chemical reagents and solvents were used as received from commercial sources unless otherwise stated. UV-Vis spectra were recorded in a 5-mm path quartz cell on a Beckman coulter DU800 spectrometer. Fluorescence spectroscopic studies were

carried out on Varian Cary Eclipse Fluorescent Spectrometer. Fluorescence imaging was acquired with a confocal fluorescence microscope (Nikon Eclipse TE2000-E), using a super high pressure mercury lamp (Nikon, TE2-PS100W) with an excitation filter: 535/50 nm and emission filter: 610/75 nm. High performance liquid chromatography (HPLC) was performed on a reverse-phase column with a Shimadzu HPLC system. Analytical reverse-phase high performance liquid chromatography (RP-HPLC) was performed on Alltima C-18 column (250×3.0 mm) at a flow rate of 1.0 mL/min and semi-preparative RP-HPLC was performed on the similar C-18 column (250×10 mm) at a flow rate of 3 mL/min. HPLC elution employed linear gradients of [0.1% trifluoroacetic acid (TFA) in water (solution A)] and [0.1% TFA in acetonitrile (solution B)]. The linear gradient started from 70% solution A and 30% solution B, changed to 68.5% solution A and 31.5% solution B in 18 min, and to 0% solution A and 100% solution B in the following 12 min, and then to 70% solution A and 30% solution B in the next 5 min. ¹H NMR spectra were obtained on a 300 MHz Bruker Advance in DMSO-*d*₆. MALDI-MS spectrometric analyses were performed at the Mass Spectrometry Facility of School of Biological Sciences, Nanyang Technological University, Singapore.

3.2.2 Synthesis of monovalent Van-porphyrin 3a

Vancomycin hydrochloride (52.8 mg, 35.9 μmol, 1.04 equiv.) and porphyrin **2** (20.8 mg, 35.1 μmol, 1.00 equiv.)²⁷ were dissolved in 2 mL of dry dimethyl sulfoxide (DMSO). The mixture was cooled to 0 °C, and *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) (38.0 mg, 100.2 μmol, 2.85 equiv.)

in 1 mL of dry *N,N*-dimethylformamide (DMF) was added, followed by *N,N*-diisopropylethylamine (DIPEA) (0.06 mL, 344 μ mol, 9.8 equiv.). The mixture was allowed to rise to room temperature and stirred overnight. The reaction was quenched by adding dropwise 20 mL of acetone. A deep purple solid was precipitated, filtered out and was washed once by 5 mL of acetone. The crude product was purified by reversed-phase HPLC (RP-HPLC) to give 37.4 mg (18.5 μ mol) of pure product (yield: 52.9%). ¹H-NMR (300 MHz, DMSO-*d*₆): 10.28 (br s), 10.22 (s), 10.27 (s), 9.43 (br s), 9.12 (br s), 8.68 (br s), 8.60 (br s), 8.18 (s), 7.97 (s), 7.79 (d, 8.6 Hz), 7.50-7.55 (overlapped), 7.28-7.40 (overlapped), 6.82 (s), 6.62 (s), 6.45 (s), 6.09 (br s), 5.81 (br s), 5.57 (s), 5.39 (s), 5.27 (s), 5.18 (br s), 4.90 (br s), 4.70 (d, 5.7 Hz), 4.30-4.38 (overlapped), 4.03-4.14 (overlapped), 3.70 (m), 2.88 (s), 2.69 (s), 2.38 (s), 2.26 (m), 1.91 (s), 1.70 (m), 1.54 (m), 1.35 (s), 1.10 (m), 0.85-0.95 (overlapped). MALDI-TOF-MS: The peaks at *m/z* 2025 correspond to *M*⁺, respectively.

3.2.3 Synthesis of divalent Van-porphyrin 3b

Vancomycin hydrochloride (106.5 mg, 71.7 μ mol, 2.04 equiv.) and **2** (20.8 mg, 35.1 μ mol, 1.00 equiv.) were dissolved in 2 mL of dry dimethyl sulfoxide (DMSO). The mixture was cooled to 0 °C, and *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) (38.0 mg, 100.2 μ mol, 2.85 equiv.) in 1 mL of dry *N,N*-dimethylformamide (DMF) was added, followed by *N,N*-diisopropylethylamine (DIPEA) (0.06 mL, 344 μ mol, 9.8 equiv.). The mixture was allowed to rise to room temperature and stirred overnight. The reaction was quenched

by adding dropwise 20 mL of acetone. A deep purple solid was precipitated, filtered out and was washed once by 5 mL of acetone. The crude product was purified by reversed-phase HPLC (RP-HPLC) to give 64.9 mg (18.8 μ mol) of pure product (yield: 53.6%). $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$): 8.98 (br s), 8.69 (br s), 7.94 (s), 7.67 (br s), 7.54 (br s), 7.28 (d, 8.4 Hz), 6.92 (d, 8.0 Hz), 6.80 (d, 8.1 Hz), 6.64 (br s), 6.54 (s), 6.43 (s), 6.37 (s), 5.96 (br s), 5.78 (br s), 5.62 (br s), 5.39 (br s), 5.23 (br s), 5.18 (br s), 4.93 (br m), 4.66 (d, 5.7 Hz), 4.23 (overlapped), 4.06 (br m), 3.80 (br m), 3.69 (overlapped), 3.27 (br s), 3.04-2.88 (multiple), 2.63 (s), 2.28-2.10 (overlapped), 1.91-1.69 (overlapped), 1.61-1.56 (multiple), 1.30 (s), 1.12 (t, 13.6 Hz), 0.94 (d, 6.4Hz), 0.88 (d, 5.5 Hz). MALDI-TOF-MS: calc. $M^+ = 3455.3$, obsvd., the peaks at m/z 3455 correspond to M^+ , respectively.

In order to ascertain that the porphyrin is covalently bonded to Van, both **3a** and **3b** were effectively separated by reverse-phase HPLC and both molecules show characteristic retention times. On the other hand, a mixture consisting of porphyrin and Van shows only the retention times of the individual species. This result rules out the possibility of non-covalent adduct formation between Van and porphyrin. In addition, the MALDI-TOF-MS results clearly yield the precise mass of **3a** and **3b**, indicating the formation of covalently Van bonded porphyrin moieties.

3.2.4 UV-Vis and Fluorescence Detection

Stock solution (2 mM in DMSO) of porphyrin (**2**), monovalent (**3a**) and divalent (**3b**) Van-porphyrin were diluted to 20 μ M in 10 mM PBS, pH 7.2, containing 1.0% DMSO as co-solvent. 200 μ M vancomycin was also prepared in 10 mM PBS, pH =

7.2. Solution of each compound (600 μ L) was added into a 5 mm path quartz cell and the UV-Vis spectra were recorded using a Beckman coulter DU800 spectrometer. Fluorescence spectroscopic studies were also performed using a Varian Cary Eclipse Fluorescence Spectrophotometer.²⁸

3.2.5 Measurements of singlet oxygen ($^1\text{O}_2$) generation

Porphyrin (**2**), monovalent (**3a**) and divalent (**3b**) Van-porphyrin (10 μ M) were mixed with 9, 10-anthracenediyl-bis(methylene) dimalonic acid (ABDA) (20 μ M) in PBS buffer (10 mM, pH 7.2) and placed in quartz cuvette. The sample solutions were illuminated with white light (400 nm - 800 nm) isolated from the emission of a Xenon lamp for 2 min and then the fluorescent emission of ABDA was measured at 431 nm when excited at 380 nm. The same sample solutions without light irradiation were used as control. The destruction of ABDA indicated the generation of singlet oxygen.²⁹

3.2.6 MIC test

A standard broth dilution method was used to determine the MICs.³⁰ Porphyrin (**2**), Van-porphyrin monovalent (**3a**) and divalent (**3b**) were dissolved in DMSO to obtain 4 mg/mL stock solution and 4 mg/mL vancomycin/H₂O stock solution was also prepared. A total of 250 μ L of LB solution was added to a series of sterile test tubes, with an additional 218 μ L added to the first one. Compound stock solution (32 μ L, 4 mg/mL) was added to the first test tube, and a series of 2-fold dilutions were prepared by transferring 250 μ L to successive tubes. A 5 mL culture of three bacterial strains,

Bacillus subtilis (ATCC 33677) *Enterococcus faecium* (ATCC 51559, VanA) and *Enterococcus faecalis* (ATCC 51299, VanB), was grown to an OD600 of 0.5 in LB medium. A 10 μ L bacterial solution was added to each tube containing different concentration of compounds. The final concentration of bacterial strains was 10^6 colony forming units (cfu) per mL. The compound-treated cultures were incubated at 37°C for 24 h, and the OD600 was measured. The reported MICs were the lowest concentrations of compounds that prevented cell growth. Each measurement was performed in duplicate.

3.2.6 Imaging tests

Single colonies of *Escherichia coli* (ATCC 53868), *Bacillus subtilis* (ATCC 33677) *Enterococcus faecium* (ATCC 51559, VanA) and *Enterococcus faecalis* (ATCC 51299, VanB) on solid Luria-Bertani (LB) plates were transferred to 5 mL of liquid LB culture medium and were grown at 37 °C for 12 hr. Bacteria were harvested by centrifuging (4000 rpm for 10 min) and washed with sterile phosphate-buffered saline (PBS) three times. The supernatant was discarded and the remaining bacteria were re-suspended in PBS with an OD600 of 0.5. Then, 2 μ M or 10 μ M of porphyrin (**2**), monovalent Van-porphyrin (**3a**) and divalent Van-porphyrin (**3b**) were added to bacterial cells suspensions and incubated in the dark for 1 hr at 37 °C. After three times PBS washing, bacterial cells were spotted on glass slides and immobilized by the coverslips. Cell imaging tests were conducted with a Nikon Eclipse TE2000 Confocal Microscope. Images were captured with CFI VC 100 $\times$ oil immersed optics.

3.2.7 Evaluation of photodynamic inactivation of bacterial strains

Photodynamic treatment was performed according to the methods previously described.³¹ Four bacterial strains, vancomycin-susceptible Gram-positive *Bacillus subtilis* (ATCC 33677), vancomycin-resistant *Enterococcus faecium* (ATCC 51559, VanA) and *Enterococcus faecalis* (ATCC 51299, VanB) and Gram-negative *Escherichia coli* (ATCC 53868), were used to evaluate the photodynamic killing of porphyrin (**2**), Van-porphyrin monovalent (**3a**) and divalent (**3b**). A single colony of bacteria was transferred to 5 mL of LB solution in the presence of 50 µg/mL ampicillin and was grown at 37 °C for 12 hr. Then bacterial solutions were centrifuged at 4000 rpm for 10 min at 4 °C. After washing with PBS three times, the bacteria were re-suspended in PBS with an OD600 of 0.5. Then, cells were incubated with different concentrations of **2**, **3a** and **3b** in the dark for 15 min at 37 °C. All samples were illuminated with white light (400-800 nm) isolated from the emission of a Xenon lamp. The time of illumination was adjusted from 0 to 200 s, corresponding to the total light doses from 0 to 100 J/cm². Following irradiation, bacterial suspensions were centrifuged (4000 rpm for 10 min, at 4 °C) and the supernatant was removed. After that, bacterial pellet was suspended and serially diluted (6×10⁴-fold) in PBS. A 100 µL portion of the diluted bacterial cells was spread on the solid LB agar plate and incubated for 16 hr at 37 °C. The colonies formed were counted. The percentage of dead bacteria was evaluated by dividing the number of colony-forming units (cfu) between the samples incubated with photosensitizers and the control without photosensitizers and light exposure treatment.

3.3 Results and discussions

3.3.1 Synthesis and characterizations of vancomycin derivatives

Scheme 3.2 illustrates the synthetic pathway for preparing the divalent Van derivative. Typically, the commercially available Van (**1**) reacted with porphyrin derivative (**2**), to afford Van carboxamide (**3b**) by employing HBTU as the coupling reagent. The divalent conjugate was purified in 53.6 % yield by reversed phase HPLC and characterized by ¹H NMR spectroscopy and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Similarly, the monovalent Van adduct (**3a**) with porphyrin was also prepared in 52.9 % yield by using excess amount of porphyrin. After obtaining the Van-porphyrin derivatives (**3a-b**), their photochemical properties were investigated. Figure 3.3 shows their UV-Visible and fluorescence spectroscopy, which indicated that the monovalent and divalent Van derivatives exhibited absorption bands of both Van (~280 nm) and porphyrin moieties (B band around 400 nm, Q bands between 500 and 620 nm). The emission spectra of the Van- porphyrin precursors showed no difference from those of porphyrin molecules, suggesting Van conjugations have no effect on the fluorescent property of porphyrin. Moreover, all the precursors could produce singlet oxygen upon white light illumination (data not shown).

Scheme 3.2. The structures and synthetic scheme of Van-porphyrin derivatives.

Figure 3.3. Absorption (a) and fluorescence (b) spectra of 1, 2, 3a and 3b in PBS (1% DMSO) at room temperature. ($\lambda_{\text{ex}} = 530 \text{ nm}$).

3.3.2 *In vitro* antibacterial activities of vancomycin derivatives

The *in vitro* antibacterial activities of Van-porphyrins were first investigated by standard broth microdilution assays.³⁻¹⁰ In a typical study, three bacterial strains:

Van-sensitive strain, *Bacillus subtilis* (ATCC 33677) and two Van resistant *enterococci* (VRE) including *Enterococcus faecium* (VanA genotype, ATCC 51559) and *Enterococcus faecalis* (VanB genotype, ATCC 51299) were chosen as model organisms. Both monovalent (**3a**) and divalent (**3b**) Van derivatives showed effective MIC activity against Van sensitive *B. subtilis* which was similar to the parent Van molecule (Table 3.1). However, **3a** and **3b** demonstrated distinct decrease in their antimicrobial activities against VRE (Table 3.1). The exact nature of mechanism regarding the low activity of **3b** is unclear at this moment. Although previous studies indicated the enhanced affinities of divalent Vans for the bacterial cell wall precursors,³⁻¹⁰ the binding affinity between Van derivatives and cell wall precursors may not correlate well with the potent MIC activity against VRE.^{11,12}

Table 3.1. The antibacterial activities (MIC) of vancomycin and vancomycin-porphyrin conjugates.

Compds	<i>B. subtilis</i> (sensitive)	MIC	
		<i>E. Faecium</i> (VanA)	<i>E. Faecalis</i> (VanB)
1	1 μ M (1.5 μ g/mL)	>88.3 μ M (128 μ g/mL)	44.2 μ M (64 μ g/mL)
3a	3 μ M (6 μ g/mL)	>15.8 μ M (32 μ g/mL)	>15.8 μ M (32 μ g/mL)
3b	2 μ M (7 μ g/mL)	>18.5 μ M (64 μ g/mL)	>18.5 μ M (64 μ g/mL)

^aThe MIC values of the substrate for bacterial strains VanA and VanB cannot be further determined due to the low solubility in LB medium.

3.3.3 Bacterial fluorescent imaging of vancomycin derivatives

The binding affinity of Van-porphyrins towards various bacteria was further identified by fluorescent imaging technique. Typically, the bacterial strains were incubated with Van-porphyrin derivatives at 37 °C for 1 hour in culture media. After the cells were washed three times with PBS buffer to remove the unbound Van-porphyrins, the bacterial imaging was conducted upon the excitation of the Q bands of porphyrin under fluorescent microscope.³¹ As shown in Figure 3.4(c), incubation of porphyrin (**2**) itself with *B. subtilis* would not lead to obvious fluorescence. However, upon the specific targeting of Van affinity ligand, both **3a** and **3b** (2 µM) revealed obvious fluorescent signals in *B. subtilis* (Figure 3.4(a) and (b)). Compared to **3a**, **3b** exhibited stronger fluorescence, suggesting the higher binding association of **3b** to the surface of *B. subtilis*. Similar bacterial imaging was also carried out by incubating VRE with 2 µM of **3a** and **3b**, separately. There was no significant fluorescence observed in these strains (Figure 3.5) and the effective fluorescent imaging could only be detected when a higher concentration of **3b** (10 µM) was used (Figure 3.4(d) and (g)), indicating the lower binding affinity of Van-porphyrins to the bacterial cell walls of VRE as compared to Van-sensitive bacterial strain. However, when compared to **3a**, the multivalent/polyvalent interactions found in the divalent Van-porphyrin (**3b**) significantly improved the association between **3b** and the drug resistance bacteria. In addition, Figure 3.4 shows that incubation of **3b** with VanA type VRE (Figure 3.4(d)) displayed a lower fluorescent signal as compared to **3b** incubated with VanB (Figure 3.4(g)). This

suggested a higher affinity between the divalent derivative and VanB strain. There was no obvious fluorescent signal observed in control *E. coli* imaging experiment indicating the lowest binding affinity between Van derivatives and Gram-negative strain (data not shown).

Figure 3.4. Fluorescent imaging of bacterial staining with Van-porphyrin derivatives.

(a) - (c), *B. subtilis* loaded with 2 μ M of **3b**, **3a**, and **2**, respectively; (d) - (f), *E. faecium* (VanA) with 10 μ M of **3b**, **3a**, and **2**; (g) - (i), *E. faecalis* (VanB) same as VanA. Ex = 535/50 nm; Em = 610/75 nm.

Figure 3.5. Fluorescent imaging of bacterial staining with Van-porphyrin derivatives.

(a) - (c), *E. faecium* (VanA) loaded with 2 μ M of **3b**, **3a**, and **2**; (d) - (f), *E. faecalis* (VanB) loaded with 2 μ M of **3b**, **3a**, and **2**. Ex = 535/50 nm; Em = 610/75 nm.

3.3.4 Photodynamic antibacterial activities of vancomycin derivatives

In order to further explore the photodynamic inactivation of VRE by Van-porphyrins, the PACT treatment was performed in the dark and upon white light exposure by a traditional surface plating approach.^{17,31} In this study, photosensitizers including **2**, **3a** and **3b** were incubated with VanA and VanB, separately. Upon white light irradiation, the bacteria lethality was evaluated by counting the numbers of colony forming units (cfu) on LB agar plate. Figure 3.6 displayed the bacterial lethality of VRE under different photosensitizer concentrations. It was found that increasing concentrations of photosensitizers enhanced the bacterial killing efficiency for both VanA and VanB. Among the three photosensitizers used, **3b** showed the highest antibacterial activity against VRE throughout the whole concentration range. About 95% bacterial lethality could be observed in **3b** (2 μ M) incubated VanB upon

irradiation with 60 J/cm^2 of white light, whereas, a smaller killing efficiency ($\sim 66\%$) was detected for VanA suspension when exposed to the same dose of light. The photodynamic inactivation of both VanA and VanB were further investigated in the presence of different doses of white light (Figure 3.7) while maintaining a fixed concentration ($2 \text{ }\mu\text{M}$) of photosensitizers. Light irradiation of both VRE strains but no photosensitizers incubation would not induce obvious bacterial damage which was used as the control. There was no significant bacterial lethality detected for **2** incubated VanA and VanB strains upon light exposure. On the other hand, **3a** and **3b** revealed the effective photodynamic inactivation of VanA and VanB upon exposing the bacteria to different doses of light and more significant bacterial reduction (e.g. $> 99\%$) could be achieved when higher doses of irradiation was applied (Figure 3.7). This clearly showed that Van acted as an efficient affinity ligand and aided in targeting the porphyrin moiety to the VRE surfaces which resulted in an effective drug resistant bacterial lethality upon PACT treatment. Compared to **3a**, **3b** displayed substantially enhanced potency against VRE. The significant bacterial lethality achieved for VanA ($\sim 66\%$) and VanB ($\sim 95\%$) when $2 \text{ }\mu\text{M}$ of **3b** was incubated with VRE strains and irradiated with 60 J/cm^2 of white light was more potent than the MIC values of Van itself on VanA (~ 44 times) and VanB (~ 22 times) separately (Table 3.1). Moreover, the photodynamic inactivation was also carried out by incubating *B. subtilis* and *E. coli* with different concentrations of **2**, **3a** and **3b**. Similarly, **3b** displayed the highest potency against *B. subtilis* among the three photosensitizers. More than 95% bacterial lethality was observed when $0.5 \text{ }\mu\text{M}$ **3b** incubated bacteria

was exposed to 60 J/cm^2 of white light, which was more effective (~ 4 times) than the value of **3b** in MIC measurements (Table 3.1). There was almost no lethality observed in *E. coli* for **2**, **3a** and **3b** (Figure 3.8). These results unequivocally demonstrated that porphyrin conjugated divalent Van could serve as an effective photoactive antibacterial reagent against Van-sensitive and VRE strains due to the stronger association between **3b** and the bacteria as a result of efficient multivalent/polyvalent interactions. This is consistent with the results observed in the bacterial imaging measurements.

Figure 3.6. Photodynamic inactivation of bacterial strains towards different concentration of compounds **2**, **3a** and **3b**. (a): *E. faecium* (VanA); (b): *E. faecalis* (VanB). The white-light dose was 60 J/cm^2 (exposure for 2 min at a fluence rate of 500 mW/cm^2). Bacteria treated with compound **3b** but no light illumination as control groups.

Figure 3.7. Light dose-dependent bacterial lethality towards different compounds **2**, **3a** and **3b**. (a): *E. faecium* (VanA); (b): *E. faecalis* (VanB). The concentration of all compounds was 2 μM. Bacteria treated with light illumination (without any photosensitizers) as control groups.

Figure 3.8. Photodynamic antibacterial activity towards *E. coli* in the presence of compounds **2**, **3a** and **3b**. (a) Bacterial lethality with incubation of different concentration of compounds **2**, **3a** and **3b**. The white-light dose was 60 J/cm². Bacteria treated with compound **3b** but no light illumination as control groups. (b) Light dose-dependent bacterial lethality with incubation of 2 μM **2**, **3a** and **3b** in the presence of different doses of white light irradiation. *E. coli* treated with light illumination without any compound incubation was used as control group.

Figure 3.9. Photodynamic antibacterial activity towards *B. subtilis* in the presence of compounds **2**, **3a** and **3b**. (a) bacterial lethality with incubation of different concentration of compounds **2**, **3a** and **3b** in the dark. (b) bacterial lethality with incubation of different concentration of compounds **2**, **3a** and **3b** upon 60 J/cm² of white light illumination. (c) Light dose-dependent bacterial lethality with incubation of 0.5 μM **2**, **3a** and **3b** in the presence of different doses of white light irradiation. *B. subtilis* treated with light illumination without any compound incubation was used as control group.

3.4 Conclusion

In summary, this work presents a simple and novel phototherapeutic reagent by conjugating the photosensitizer, porphyrin with two Van moieties. This divalent Van-porphyrin exhibits a relatively higher binding affinity to bacterial surface and retains potent PACT activities against vancomycin-sensitive and VRE bacteria when compared to Van and porphyrin alone. Apart from the enhanced photodynamic antimicrobial activity, the red fluorescent emission of Van-porphyrin conjugate can be used to carry out noninvasive imaging study in living bacterial strains. One possible limitation to influence the efficacy of PACT *in vivo* could be light penetration to the site of infection. However, the effective light delivery to target tissue could be achieved with advent of fiber optics, catheters and two-photon excitation laser system.³²⁻³⁴ So far, photodynamic therapy based on some porphyrin photosensitizers has obtained clinical approval in many countries for treating various types of diseases.^{14,15} We expect that this multifunctional divalent vancomycin provides the possibilities for photodynamic inactivation of antibiotic-resistant bacteria. It may also act as a useful fluorescent probe to image bacteria or other cells in an effective manner.

3.5 Reference

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Chapter 4

Presenting vancomycin on SERS nanoparticles for Bacterial Raman imaging

4.1 Introduction

Vancomycin (Van), as a glycopeptide antibiotic, is initially found to be used as an antibacterial drug through forming hydrogen bindings with Gram-positive bacteria cell walls, which resist their biosynthesis and induce bacterial death.^{1,2} In virtue of the highly specific and extremely strong binding (dissociation constant around 1 μM),³⁻⁵ vancomycin monomer has also been employed as an affinity ligand of fluorescent probe for optical bacterial imaging. Extensive research works on fluorescent bacterial imaging have been published. Errington *et al.* used a fluorescent derivative of vancomycin to explore nascent peptidoglycan synthesis of various Gram-positive bacteria, revealing the ways to control cell morphogenesis in bacteria.⁶ A similar work by Rudner and Walker has also been reported, in which fluorescent labeled antibiotics (vancomycin and ramoplanin) served as a probe to image the structure of peptidoglycan in *Bacillus subtilis*.⁷ Moreover, Pinho's group also employed fluorescent labeled vancomycin derivative, combined with fluorescence ratio imaging microscopy, to investigate the mechanism of vancomycin resistance and to establish the molecular models of vancomycin resistant *staphylococcus aureus*.⁸ Recently, based on the multivalent interaction between vancomycin dimmers and Gram-positive bacteria cell walls, our group designed a divalent vancomycin-porphyrin conjugate to

improve its property in fluorescent imaging of Gram-positive bacteria, especially vancomycin resistant bacterial strains.⁹

Although considerable studies have been reported based on vancomycin conjugated fluorescent probes, there are some intrinsic problems of fluorescence based materials, such as photobleaching, narrow excitation with broad emission profiles and peak overlapping in multiplex experiments. In order to address these issues, surface enhanced Raman scattering (SERS) probes have been developed recently.^{10,11} SERS is a surface-sensitive technique that offers orders of magnitude enhancement in Raman intensity through molecules adsorbed on rough metal surfaces. Based on SERS technology, 10^6 - 10^{14} folds increase of Raman signal intensity could be provided, which is sufficient to detect pico- to femtomolar amounts of biomolecules.^{12,13} Moreover, compared to fluorescent labels, the SERS tagging is resistant to photo-bleaching and could be excited by multi light sources (especially near infrared light for human tissue), giving narrow peak widths to avoid spectral overlapping.^{14,15} Therefore, SERS tags have been attracted considerable attentions as a class of emerging labels for biomedical systems.^{16,17}

Among optical enhancement substrates for SERS tags, colloidal gold nanoparticles show excellent properties: long-term stability, controllable size distribution and high homogeneity.¹⁸ Conversely, it gives only around 1/100 SERS intensity from silver nanoparticles (AgNPs) of similar size,¹⁹ indicating that AgNPs could be a better choice to produce SERS signals. AgNPs are well-known to be very unstable since they are limited soluble and easy to aggregate.²⁰ In order to overcome the drawbacks

of both gold and silver nanoparticles, silver coated gold nanoparticles ($\text{Au}@\text{AgNPs}$) were prepared for the optical enhancement nanoparticles of SERS tags.²¹

In this chapter, a SERS tag for bacterial Raman imaging was designed, in which $\text{Au}@\text{AgNPs}$ were chose to serve as Raman enhancement substrate and silica shell was functionalized with vancomycin as binding affinity groups for bacterial cell walls. (Scheme 4.1) This core-shell SERS tag was synthesized through a rapid and straightforward method, in which core $\text{Au}@\text{AgNPs}$ can be directly coated by a sol-gel procedure after the adsorption of Raman reporter molecules (4-Mercaptobenzoic acid (4-MBA)). After bio-functionalized with vancomycin linkers, further investigations were performed to evaluate its property in Raman imaging of drug sensitive and resistant bacterial strains.

Scheme 4.1. Synthesis route of vancomycin modified SERS tag. Raman reporter is

4-Mercaptobenzoic acid (4-MBA).

4.2 Experimental Sections

4.2.1 General

Chemical reagents and solvents were used as received from commercial sources unless otherwise stated. ¹H NMR spectra were obtained on a 300 MHz Bruker Advance in CDCl₃. MALDI-MS spectrometric analysis was performed at the mass spectrometry facility of Division of Chemistry and Biological Chemistry.

4.2.2 Synthesis of Van-linker-osu

Synthesis of compound **1**: glutaric acid (300 mg, 2.27 mmol, 1 eq) and N-hydroxysuccinimide (575 mg, 4.59 mmol, 2.2 eq) were dissolved in 2 mL DMF at 0 °C. DMAP (28 mg, 0.23 mmol, 0.1 eq) and EDC (713 mg, 4.59 mmol, 2.2 eq) were added subsequently. The mixture was allowed to react overnight and then added into cold diethyl ether to obtain precipitate. The product was then washed by EA and water and extracted with Brine twice. The crude product was dried with MgSO₄ and purified with flash chromatography with eluent EA and Hexane (2:1) to afford white precipitate **2** (444 mg, 60%). ¹H NMR (300 MHz; CDCl₃, 25°C): δ=2.147-2.243 (2H, m), 2.775- 2.842 (12H, m).

Synthesis of compound **2**: vancomycin (20 mg, 0.0135 mmol, 1 eq) and compound **1** (23 mg, 0.0705 mmol, 5 eq) were dissolved into 2 mL DMF. The mixture was cooled into 0 °C, and 10 µL DIPEA was then added. The mixture was allowed to rise to room temperature and stirred overnight. The reaction was quenched by adding dropwise 50 mL acetone. A white solid was precipitated and weight 20.3 mg.

MALDI-TOF-MS: The peak at m/z 1661 correspond to M^+ , respectively.

4.2.3 Preparation of silver coated gold nanoparticle (Au@AgNPs)

Au@AgNPs (~ 60 nm) was synthesized through the method reported before:²² In 50 mL pure water, HAuCl_4 (82.2 μL , 0.01%) was added and heated for 30 min at 100 $^\circ\text{C}$ under vigorous magnetic stirring. Then, sodium citrate (500 μL , 1% w/w) was added and heated for 15min. After that, AgNO_3 (235 μL , 0.1 M) was dropped into the boiling solution and subsequently, sodium citrate (800 μL , 1% w/w) was added dropwise. The solution was kept boiling for 1 hr resulting in an orange-colored Au@Ag core-shell nanoparticles.

4.2.4 Synthesis of silica coated Au@AgNPs (Au@AgNP@SiO₂) by using 4-MBA as Raman reporter

Au@AgNPs (60 nm) was concentrated from 60 mL into 10 mL by centrifuging 5000 rpm for 10 min. Then, particles were slowly transferred to 50 mL 2-propanol under vigorous stirring and 200 μL 4-Mercapto Benzoic Acid (5 mM in ethanol) was added. After 10 min reaction, 12 mL TEOS (0.6%, w/w in ethanol) was added dropwise, following by the 1.8 mL ammonia addition. After 1.5 hr, the reaction was quenched through centrifuging 100,000 rpm for 15 min and particles were washed with ethanol three times.

4.2.5 Immobilization of vancomycin linker on the Au@AgNP@SiO₂ (Au@AgNP@SiO₂-Van)

APTMS (3.6 mL, 20%, w/w in ethanol) was dropped slowly into Au@AgNP@SiO₂ (0.1 nM, 180 mL in ethanol), and then 5.4 mL of ammonia was added. The resulting dispersion was stirred vigorously for 1 hr at room temperature. The nanoparticles were washed with ethanol three times and then redispersed into 1 mL dry DMSO. Van-linker-osu (**2**) (25 μ L, 10 mM) and TEA (3 μ L) were subsequently added into the APTMS treated silica coated NPs solution. After overnight reaction, the final particles were collected and washed with DMSO. Then the Au@AgNP@SiO₂-Van was dried and kept at 4 °C.

Scheme 4.2. The synthetic route of Au@AgNP@SiO₂-Van.

4.2.6 Transmission electron microscopy (TEM) for particle size analysis

All the TEM photographs were taken on JEOL 2000 EX transmission electron microscope at 100 kV. Nanoparticles solution was dropped onto the carbon-coated copper grids (200 mesh). Then the nanoparticles solution was allowed to settle on grids for 30 mins before the excess solution was wicked away with filter paper.

4.2.7 Dynamic light scattering (DLS) for particle size analysis

The size population distributions of Au@AgNPs and Au@AgNP@SiO₂ were determined on a Brookhaven Instruments spectrophotometer. All the measurements were performed at an angle of 90 under room temperature. The CONTIN algorithm was used for analyze the DLS data.

4.2.8 UV-Vis and Raman detection

Au@AgNPs, Au@AgNP@SiO₂-NH₂ and Au@AgNP@SiO₂-Van were dissolved in water. Vancomycin (200 μ M) was also prepared in water. Each solution (600 μ L) was added into 5-mm path quartz cell and the UV-Vis spectra were recorded using a NanoDrop 2000c Spectrophotometer (Thermo Scientific).

Au@AgNPs and Au@AgNP@SiO₂ were dissolved in pure water. Their Raman spectra were collected from the sample solution on an R-3000HR spectrometer (Raman system, Inc, R-3000 series) with a red-light-emitting diode (LED) laser (λ = 785nm) at 290 mW.

4.2.9 Bacteria culture

Four bacterial strains, Gram-positive vancomycin-sensitive *Bacillus subtilis* (ATCC33677), vancomycin-resistant *Enterococcus faecium* (ATCC 51559, VanA), *Enterococcus faecalis* (ATCC 51299, VanB) and Gram-negative *Escherichia coli* (ATCC 53868), were culture in 5 mL of liquid LB medium at 37 °C for 12 hrs. Before incubated with nanoparticles, all bacteria were washed with 10 mM phosphate buffer (pH 7.4) three times and were adjusted to a concentration of 10^8 cfu/mL.

4.2.10 Minimal inhibition concentration (MIC) tests

The antibacterial activities of SERS tags were investigated through the broth microdilution assays with some modifications. Au@AgNP@SiO₂-NH₂ and Au@AgNP@SiO₂-Van were dissolved in sodium phosphate buffer (10 mM) to obtain 0.4 nM stock solutions and 10 µM vancomycin was also prepared. A total of 500 µL of stock solutions was added to the first test tube, and a series of 2-fold dilutions were prepared by addition of 500 µL 10 mM sodium phosphate buffer, pH 7.4. A 10 µL different bacterial solution was incubated with the nanoparticle solutions of different concentrations. The final concentration of bacterial strains was 10^6 cfu/mL. After 1 hr incubation at 37 °C, LB buffer (500 µL) was added and incubated at 37 °C for 24 hrs. The reported MICs were the lowest concentrations of nanoparticles that prevented bacterial growth. Each measurement was performed in duplicate.

4.2.11 Bacterial tests using solution Raman Spectroscopy

Au@AgNP@SiO₂-NH₂ and Au@AgNP@SiO₂-Van (500 μ L, 60 pM in phosphate buffer, pH 7.4) were incubated with different bacterial strains (final concentration 10⁸ cfu/mL). After 1 hr incubation at 37 °C, bacterial cells were separated with free nanoparticles and then suspended with 500 μ L buffer. The Raman spectra were collected by using the IR-3000 system with 785 nm laser source (290 mW).

4.2.12 Bacterial Raman imaging with confocal Raman microscopy

Au@AgNP@SiO₂-NH₂ or Au@AgNP@SiO₂-Van (500 μ L, 60 pM in phosphate buffer) were incubated with different bacterial strains (10⁸ cfu/mL). After 1 hr incubation at 37 °C, bacteria were separated with free nanoparticles and then suspended with 500 μ L buffer. About 10 μ L of Raman tags treated bacterial solution was dropped onto polylysine-coated coverslips and dried on air. The Raman mapping was acquired using a Raman microscope (HARIBA, LabRaman HR). A 632.8 nm output from a He-Ne laser source was passed through an interference filter to eliminate the plasma line of the laser. A motorized XY stage was utilized to perform Raman mapping. The laser power at the sample was 2 mW and Raman signal was collected with 100 $\times$ objective (NA 0.9), passed through a notch filter to reject the Rayleigh line and directed through a 500 μ m confocal hole, then dispersed by a 600 grooves/mm grating and detected by a CCD. Calibration was done referring to 0 nm using zeroth order white light and then to 520 cm⁻¹ line of silicon. All spectra were processed via the LabSpec 5 software which controlled the instrument itself including

data acquisition.

4.3 Results and Discussion

4.3.1 Synthesis and characterizations of SERS bio-tags

Silver coated gold nanoparticles were synthesized according to the methods reported previously with some minor modifications.²² The hydrodynamic diameter and size distribution of the synthesized silver coated gold nanoparticles were characterized by laser dynamic light scattering (DLS) and transmission electron microscopy (TEM) (Figure 4.3). The results indicated the narrow size distribution of Au@AgNPs with an overall diameter of 60 nm (Figure 4.3(a)), which was proved to be the most suitable size for surface enhanced Raman detection.¹⁹ Raman molecule, 4-mercaptobenzoic acid (4-MBA), was chosen to serve as the source of Raman enhancement. 4-mercaptobenzoic acid is the simplest aromatic compound containing both a thiol group and a carboxylic acid group. This compound could replace the stabilizer (sodium citrate) of silver-gold core shell nanoparticles and attach onto the surface of nanoparticles through the formation of stable Ag-S covalent bonds between the thiol groups and nanoparticles. The MBA labeled nanoparticles were further coated with silane based on Stöber method²³. The hydroxyl group of 4-MBA rendered silica deposited onto the Au@AgNPs by the base-catalyzed hydrolysis of tetraethyl orthosilicate, subsequently condensed to form a core-shell structure,²⁴ which facilitated the stabilization of the nanoparticles suspension in biological system with different pHs and enabled the functional groups' modification for further practical

applications. DLS and TEM results (Figure 4.3) showed highly uniform silica encapsulated silver coated gold nanoparticles with diameter of ~ 100 nm.

After that, the primary amino groups were introduced onto the surface of Au@AgNP@SiO_2 by a reaction with APTMS, subsequently conjugated with vancomycin linkers. The loading amount of vancomycin linker on the surface of Au@AgNP@SiO_2 was around 1500 vancomycin molecules/particle through the determination by the difference of UV-Vis absorbance spectrum (Extinction coefficient = $5587.76 \text{ cm}^{-1} \text{ M}^{-1}$) (Figure 4.4).

Figure 4.3. The size distribution of synthesized nanoparticles determined by TEM image (above) and dynamic light scattering (down) (scale bar = 100 nm). (a) Au@AgNPs ; (b) Au@AgNP@SiO_2 .

Figure 4.4. UV-Vis absorbance of vancomycin linker before and after reaction.

As shown in Figure 4.5, there is no Raman signals shown in Au@AgNPs solution, while, after 4-MBA attachment and silica encapsulation, nanoparticles show two specific Raman peaks (1075 cm^{-1} and 1585 cm^{-1}), which are the characteristic Raman bands of pure MBA molecule. After vancomycin conjugation, there is no Raman signal changed for Au@AgNP@SiO₂-Van, which demonstrated that this nanoparticle complex could be used as surface enhanced Raman scattering tag for further bio-detections.

Besides, we also examined the UV-Vis spectra of different kinds of nanoparticles (Figure 4.6). In Au@AgNPs solution, two surface Plasmon resonance (SPR) bands were found at 512 nm and 370 nm, which origin from the plasmon shift of gold core and silver shell, respectively.²² The appearance of these two SPR bands indicated that silver salt reduced on the preformed gold core surface and formed the core-shell structure of Au-Ag composites, rather than reduced alone and formed a physical

mixture of the Au and Ag nanoparticles, in which two Plasmon bands from Au and Ag colloids should always be observed at ~ 530 nm and 400 nm, respectively. When Au@AgNPs were encapsulated with silica shell, a red-shift to ~ 535 nm occurred due to the increased refractive index around the colloid particles rather than the aggregation of nanoparticles.^{25,26} Although vancomycin has a UV-Vis absorbance at 280 nm, compare to Au@AgNP@SiO₂-NH₂, SERS bio-tag conjugated with vancomycin affinity groups did not show an obvious increased absorbance at 280 nm since the amount of vancomycin attached to nanoparticles seemed to be too little to produce the enhancement of UV-Vis signal. Thus, the loading amount of vancomycin linker on surface of SERS bio-tag was determined by the difference of UV-Vis absorbance spectrum before and after reaction. (shown in Figure 4.4).

Figure 4.5. Raman spectra of pure 4-MBA, Au@AgNPs, Au@AgNP@SiO₂-NH₂ and Au@AgNP@SiO₂-Van.

Figure 4.6. UV-Vis spectra of vancomycin, Au@AgNPs, Au@AgNP@SiO₂-NH₂ and Au@AgNP@SiO₂-Van in water at room temperature.

4.3.2 The antibacterial activities (MIC) of SERS bio-tags towards various bacterial strains

The *in vitro* antimicrobial properties of SERS bio-tags were evaluated by the modified broth microdilution assays. Four bacterial strains: three Gram-positive bacterial strains, *B. Subtilis*, *E. Faecium* (VanA) and *E. faecalis* (VanB) and one Gram-negative bacteria strain, *E. Coli* were chosen as model organisms. As shown in Table 4.1, Au@AgNP@SiO₂-Van shows effective antibacterial activity against vancomycin sensitive bacteria *B. Subtilis*, in which the MIC value obtained is 10⁴-fold lower than that of the parent vancomycin molecule, due to the increased binding affinity of multivalent vancomycin on the surface of SERS bio-tag. This vancomycin modified SERS nanoparticle also exhibits excellent activity against resistant bacterial strains (*E. Faecium* (VanA) and *E. faecalis* (VanB)) with similar potency, which was

agreed with the results of vancomycin capped gold nanoparticles (Au@Van) reported previously.²⁷ In Xu's work, Au@Van was synthesized through linking bis(vancomycin) cystamide on the surface of gold nanoparticles, which also exhibited effective antibacterial activity against Gram-negative bacteria, *E. Coli*. based on an unknown mechanism.²⁷ However, in our study, Au@AgNP@SiO₂-Van did not show notable activity against *E. Coli*. The exact reason is still not clear and need to be elucidated in future work. Here, we provide two supposed reasons as followed: (1) the amount of vancomycin on the surface of SERS tag is much less than that of Au@Van. (2) Silica encapsulation decreases the solubility of nanoparticles, especially in LB medium, thus producing precipitations at higher concentration (> 400 pM). As expected, no obvious bacterial inactivation were found when four bacterial strains were incubated with Au@AgNP@SiO₂-NH₂, indicating that the antimicrobial activities were due to the vancomycin molecules attached on the surface of SERS bio-tag rather than the nanoparticles themselves.

Table 4.1. The antibacterial activities (MIC) of SERS bio-tags towards various bacterial strains.

Compds	MIC			
	<i>B. Subtilis</i>	<i>E. Faecium</i> (VanA)	<i>E. faecalis</i> (VanB)	<i>E. Coli</i>
Vancomycin	1 μ M	>88 μ M	44 μ M	>88 μ M
Au@AgNP@SiO ₂ -NH ₂	>400 pM	>400 pM	>400 pM	>400 pM
Au@AgNP@SiO ₂ -Van	100 pM	200 pM	200 pM	>400 pM

4.3.3 Bacterial Raman detections by using the SERS bio-tags

The binding affinity of SERS bio-tags towards various bacteria was evaluated by solution Raman detections. In a typical experiment, Au@AgNP@SiO₂-NH₂ or Au@AgNP@SiO₂-Van (500 μ L, 60 pM in phosphate buffer, pH 7.4) was incubated with different bacterial strains (final concentration is 10⁸ cfu/mL). After interaction for 1 hr at 37 °C, bacterial strains were separated with free nanoparticles by centrifugation and then resuspended in 500 μ L buffer solution. Raman tests were conducted under Raman spectroscopy upon solution phase. As shown in Figure 4.7, compared to Au@AgNP@SiO₂-NH₂ (Figure 4.7(b)), three Gram-positive bacterial strains, *B. Subtilis*, *E. Faecium* (VanA) and *E. faecalis* (VanB) showed much higher Raman signals upon the incubation of Au@AgNP@SiO₂-Van, due to the specific targeting properties of vancomycin affinity ligands (Figure 4.7(a)). For the control bacterial strain, *E. Coli*, only little Raman intensity changed after the treatment of the SERS tag with amine groups or vancomycin affinity ligands, indicating the lowest binding affinity between vancomycin modified SERS tag and Gram-negative strain. Within three Gram-positive bacterial strains, Raman signal of sensitive *B. Subtilis* is a little bit higher than other two VRE strains, suggesting that the higher binding affinity of the SERS bio-tag to the bacterial cell walls of sensitive strain as compared to the vancomycin resistant bacterial strains.

Figure 4.7. Raman spectra of different bacterial strains (10^8 cfu/mL) treated with (a) Au@AgNP@SiO₂-Van; (b) Au@AgNP@SiO₂-NH₂; *B. Subtilis* (black); *E. coli* (red); *E. faecium* (VanA) (blue) and *E. faecalis* (VanB) (green). The spectrum in pink is collected from pure MBA.

4.3.4 Bacterial Raman imaging by using the SERS bio-tags

Bacterial Raman imaging technique was also employed to further study on the binding affinity of SERS bio-tags towards various bacteria. In a typical assay, bacterial strains were incubated with SERS-tags at 37 °C for 1 hour. The bacterial Raman mapping was conducted under the confocal Raman microscopy, which were shown in Figure 4.8. The left-hand sides of the figure were merged pictures of photographic images and SERS intensity maps of the same surface regions, based on

the area of the peak at 1075 cm^{-1} . As shown in the figure, in comparison of $\text{Au@AgNP@SiO}_2\text{-NH}_2$, $\text{Au@AgNP@SiO}_2\text{-Van}$ exhibits Raman signal towards Gram-positive bacteria including vancomycin sensitive and resistant bacterial strains, suggesting the binding association of vancomycin linkers to the cell walls of Gram-positive bacteria. Figure 4.8(a) also shows that VanA type VRE displayed a lower Raman intensity as compared to VanB strain when they are treated with $\text{Au@AgNP@SiO}_2\text{-Van}$, which means that this vancomycin modified SERS nanoparticle has a higher affinity towards VanB strain. However, almost no obvious Raman shifts were observed in control *E. Coli* imaging assays, indicating the lowest binding affinity between $\text{Au@AgNP@SiO}_2\text{-Van}$ and Gram-negative strain.

In addition, typical SERS spectra of different points of the map, labeled with colors in the merged pictures, were shown in Figure 4.8 on the right. As shown in these spectra, the SERS signals only could be observed on the surface of bacteria. The distinct differences of Raman spectra between bacteria and background indicate that $\text{Au@AgNP@SiO}_2\text{-Van}$ specific target to both vancomycin sensitive and resistant bacterial strains with a high sensitivity. This vancomycin modified SERS nanoparticle exhibits an opportunity to be served as a potential Raman probe for bacterial Raman imaging.

Figure 4.8. Left: merged pictures of photographic images and Raman mappings of different bacterial strains treated with (a) Au@AgNP@SiO₂-Van; (b) Au@AgNP@SiO₂-NH₂. The maps are constructed on the band area at 1075 cm⁻¹. Scale bar: 1 μm. Right: the original Raman spectra of five different points in the left pictures. The Raman spectra in colors are collected from the points highlighted with same color by Raman confocal microscopy. The Raman spectrum in black is collected from pure MBA.

4.4 Conclusions

In summary, this work presents a simple and rapid procedure to synthesize core-shell SERS nanoparticles. The size and shape were well characterized by TEM and DLS. UV-Vis and Raman spectra were also performed to evaluate the properties of this SERS tag. After functionalized with vancomycin linker, upon a specific binding affinity between polyvalent vancomycin and the cell walls of Gram-positive bacteria, the SERS bio-tag is promising to be served as a Raman probe in bacterial Raman imaging, especially for vancomycin sensitive and resistant bacterial strains.

4.5 Further work and perspective

Although preliminary studies have proven that vancomycin modified SERS nanoparticles could be served as Raman probe for *in vitro* Gram-positive bacteria Raman imaging, further work is still needed for more detailed investigations on *in vivo* animal assays.

4.6 Reference

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- 1 **Tingting Jiang**, Bengang Xing* and Jianhong Rao. Recent Developments of Biological Receptor Technology for Detecting Gene Expression. *Biotechnology and Genetic Engineering Reviews*. Vol.25, 41-76 (2008).
- 2, **Tingting Jiang**, [#] Rongrong Liu, [#] Xianfeng Huang, Weiling Tew and Bengang Xing*. Colorimetric Screening of Bacterial Enzyme Activity and Inhibition Based on the Aggregation of Gold Nanoparticles. *Chem. Commun.* 2009, 1972-4. ([#] contributed equally)
- 3, Qing Shao, **Tingting Jiang**, Gang Ren, Zhen Cheng and Bengang Xing*. Photoactivable Bioluminescent Probes for Imaging Luciferase Activity. *Chem. Commun.* 2009, 4028-4030.
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2, Rongrong Liu, Xianfeng Huang, **Tingting Jiang**, Bengang Xing*. Colorimetric
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