Glycosylated cationic block co-beta-peptide as antimicrobial and anti-biofilm agents against Gram-positive bacteria

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**GLYCOSYLATED CATIONIC BLOCK CO-BETA-PEPTIDE AS
ANTIMICROBIAL AND ANTI-BIOFILM AGENTS AGAINST GRAM-
POSITIVE BACTERIA**

**ZHANG KAIXI
Interdisciplinary Graduate School
HealthTech NTU**

2019

**Glycosylated cationic block co-beta-peptide as antimicrobial and anti-biofilm
agents against Gram-positive bacteria**

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**Interdisciplinary Graduate School
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A thesis submitted to the Nanyang Technological University in partial fulfillment of
the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Authorship Attribution Statement

This thesis contains material from 1 paper(s) published in the following peer-reviewed journal(s) where I was the first author.

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The contributions of the co-authors are as follows:

- Prof Mary Chan and Prof Kevin Pethe supervised and guided the overall research.
- I prepared the manuscript drafts. The manuscript was revised by Prof Mary Chan and Prof Kevin Pethe.
- I conducted the *in vitro* tests with Sheethal Reghu and Ruan Lin for the biofilm tests.
- I conducted the *in vivo* acute wound infection model with Dr Jo Thy Subramanion and Dr Damien Keogh.
- I conducted the *ex vivo* human skin experiment with Dr Michelle Turvey.
- I conducted all the rest *in vitro* and *in vivo* tests.

- Dr Du Yu, Si Zhangyong and Dr Cheerlavancha Raju synthesized the polymers. I conducted the MALDI-TOF and GPC measurements.
- Dr Liu Yang and Prof Mu Yuguang conducted the computer simulation.
- Prof Mary Chan and Prof Guillermo Bazan guided chemical synthesis.
- Prof Ren Jinghua and Prof Zhu Yabin supervised *in vivo* toxicity tests.
- Prof K.C. Tam guided the solution property study and the DLS tests.
- Dr Kalisvar Marimuthu, Dr Partha Pratim De and Dr Oon Tek Ng isolated and provided strains from local hospital TTSH and provided useful suggestions.
- Prof Duan Hongwei, Prof Liu Xuewei, Prof Robin Chi and Prof Paula Hammond participated in the supervision of the project.

18 Dec 2019

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Abstract

Antimicrobial resistance to last-resort antibiotics is a serious and chronic global problem. The treatment of bacterial infection is further hindered by the presence of biofilm and metabolically inactive persisters. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen causing high rates of mortality and morbidity. We report the synthesis of a new enantiomeric block co-beta-peptide, poly(amido-D-glucose)-*block*-poly(beta-L-lysine)) (**PDGu-*b*-PBLK**), with high yield and purity by one-shot one-pot anionic-ring opening (co)polymerization (AROP). The co-beta-peptide is bactericidal against replicating as well as biofilm and persisters MRSA, and also disperses biofilm biomass. It is active towards both community-acquired and hospital-associated MRSA strains which are resistant to multiple drugs including vancomycin and daptomycin. Its antibacterial activity is superior to vancomycin in established MRSA murine and human *ex vivo* skin infection models, with no acute *in vivo* toxicity in repeated dosing in mice at above therapeutic levels. The bacteria-activated surfactant-like effect of the copolymer, resulting from contact with bacterial envelope, induces high bactericidal activity with low toxicity and good biofilm dispersal. This new class of non-toxic molecule, effective against all bacterial sub-populations, has promising clinical potential.

Besides *Staphylococci*, biofilms of many other Gram-positive pathogens (including *Streptococci*, *Enterococci*) are closely associated with recalcitrant infections and poor clinical outcomes of standard antibiotic treatment. Moreover, many biofilm-associated infections are polymicrobial, and targeting only one specific pathogen becomes less effective. Broad-spectrum dispersal of biofilm matrix against multiple Gram-positive genera, and thereby removing the substrate for future microbial re-colonization and persistent infection, is of great interest for

next generation antibiofilm agent development. Wall teichoic acid (WTA), an anionic glycopolymer abundantly present on the cell surface of Gram-positive bacteria, is a highly accessible but underexploited target. We identified that the block co-beta-peptide **PDGu-*b*-PBLK** targets WTA of Gram-positive bacteria and potentiates the beta-lactam antibiotic oxacillin against 4 hospital associated MRSA strains. It also disperses the biofilm of all tested Gram-positive bacteria (five species across two genres). Its cationic block electrostatically interacts with anionic WTA on cell envelope, and the glycosylated block forms a non-fouling corona around the bacteria. This reduces physical interaction of bacteria with biofilm matrix, leading to biofilm dispersal. This co-beta-peptide, which is antibiotic-potentiating, and which disperses biofilms of a broad spectrum of Gram-positive pathogens by targeting a common cell envelope component, WTA, has promising potential for future development of clinical antibacterial and anti-biofilm strategies.

Chapter 1 Introduction and literature review

Section 1.1 Overview of antimicrobial resistance (AMR)

Most of the antibiotics used today are discovered during the golden era of antibiotic development from 1950s to 1970s. Over the last three decades, the discovery of new classes of antibiotics have been largely unsuccessfully, leaving an innovation gap in the pipeline of antibiotic drug development¹. This largely owes to the wrong perception that infectious disease is a “yesterday’s problem” since the golden era of antibiotics. As a result, over-adjustment of research priorities from public funds leads to a decline in R&D for new antibiotics. On the other hand, pharmaceutical companies have reallocated resources from antimicrobials to more lucrative opportunities such as oncology, as they consider antibiotics less profitable with high regulatory requirements/risks². A recent example is Novartis out-licensing the anti-infective asset despite the promising clinical data of newly identified monobactam derivative (LYS228)³. Venture capital funds to support antibiotic development is also shrinking due to the seemingly unfavourable financial returns.

Complicating with a dwindling antibacterial pipeline, bacteria has developed resistance to every class of antibiotics, including those of the last-resort. A colistin-resistant *Enterobacteriaceae* mediated by horizontal gene transfer (*mcr-1* gene) was first isolated from a pig farm of China in 2015⁴. Soon afterwards, isolates harbouring the *mcr-1* gene has been identified in animals from Vietnam⁵ and patients from European, North American, and southeast Asian locations⁶⁻⁷. This is an alarming example of the quick dissemination of antibiotic resistance globally. The World Health Organization (WHO) recently published a priority list of bacteria for which new antibiotics are urgently needed and called for action from both public funding and private sectors to develop innovative new treatments⁸. According to a high-level

study chaired by Sir Jim O’Neil, if not properly tackled now, the AMR problem is estimated to surpass cancer and become the biggest killer by 2050, resulting in 10 million deaths per year at a cumulative cost of 100 trillion USD to global economy⁹.

Antibiotic resistance involves a genotypic change in bacteria, resulting in alteration/protection of drug target, direct or indirect inactivation of drug, reduced penetration and accumulation of drug in binding sites¹⁰. Resistant bacteria are able to replicate, and not just survive, in the presence of antibiotic at a concentration which kills (for bactericidal antibiotics) or suppresses growth (for bacteriostatic antibiotics) of antibiotic-sensitive bacteria. However, bacteria could also phenotypically “tolerate” high concentrations of bactericidal antibiotics without being genetically mutated, a term we refer to as “persistence” and these sub-population of cells are called persister cells. In response to external stresses, or even stochastically without a trigger, bacteria could enter a “dormant” state with reduced cellular activity¹¹. Because most antibiotics target macromolecular machinery only essential for active replication, they are significantly less effective against dormant persister cells¹². The clinical significance of persisters has been increasingly recognised in recent years¹¹. It is closely associated with failure of antibiotic treatment, leading to recalcitrant infections¹³. In fact, clinical isolates from chronic infections (such as *E. coli* from urinary tract infection and *P. aeruginosa* from cystic fibrosis patients) commonly express higher level of persistence¹⁴. Bacteria in persister state shows increased virulence, possibly rising from the stringent response pathways that are activated in persisters¹⁵. Increased level of persistence is also linked to higher rate of resistance evolution¹⁶.

Besides planktonic phenotype, bacteria also possess a sessile lifestyle called biofilm. Biofilms are considered as the “emergent form of life”, where it is a

protected mode for bacteria to survive in hostile environments¹⁷ and reserve a “niche” of bacteria for their future mobilization and colonization¹⁸. Biofilm is defined as “aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface”¹⁹. The EPS functions as a protective shield to protect bacteria from host immune defence, antimicrobial biocides, antibiotics, toxic metallic cations and even ultraviolet radiation^{17, 20}. Due to the heterogeneous chemical and physical microenvironments in the biofilm matrix (such as carbon sources, oxygen and other nutrients), bacteria in biofilms show heterogeneous physiological states, whereby those residing deep in the biofilm become metabolically dormant persisters²¹. US National Institutes of Health has attributed more than 80% of infection in the body to be biofilm-related²². The capacity of the bacteria to establish stable biofilms is commonly associated with longer treatment time, frequent relapse and higher resistance propensity.

In summary, bacteria have evolved resistance through miscellaneous genetic pathways²³, and the presence of antibiotic tolerant persisters and biofilms further complicate the effective treatment of infectious disease and contribute to the global problem of AMR. Due to the high clinical relevance and constant failure of standard antibiotic treatment, new strategies to tackle antibiotic resistance, persisters and biofilms are urgently needed.

Section 1.2 Aims and objectives

Methicillin-resistant *Staphylococcus aureus* (MRSA), a WHO high priority pathogen, is a leading cause of mortality due to antibiotic-resistant infections²⁴⁻²⁵. MRSA is prone to form biofilms and also exists in the form of metabolically inactive

antibiotic-tolerate persisters²⁶. Last resort antibiotics such as vancomycin are largely ineffective against MRSA persisters and biofilms²⁷. New therapeutics are needed to combat the spread of difficult to treat drug-resistant MRSA infections. Hence, this thesis aims to develop novel antimicrobials to eradicate all sub-populations of antibiotic-resistant *Staphylococcus aureus* (metabolically active, persisters and biofilms). The antimicrobial should combine excellent efficacy and biocompatibility, both need to be demonstrated *in vitro* and *in vivo*.

Besides *Staphylococci*, biofilms of many other Gram-positive pathogens (such as *Streptococci*, *Enterococci*) are closely associated with recalcitrant infections and poor clinical outcomes of standard antibiotic treatment²⁸⁻²⁹. Moreover, many biofilm-associated infections are polymicrobial, and targeting only one specific pathogen becomes less effective³⁰. Hence broad-spectrum biofilm dispersal against multiple Gram-positive genera is particularly attractive. This thesis aims to shed light on a new strategy for broad-spectrum Gram-positive biofilm dispersal. The work presents in the later chapters aims to exploit a molecule that abundantly presents on the surface of Gram-positive bacteria, the wall teichoic acid (WTA), to enable the broad-spectrum biofilm dispersal effect of the block co-beta-peptide against clinically relevant Gram-positive bacteria.

This thesis aims to develop novel antimicrobial and anti-biofilm agents against Gram-positive bacteria. It consists of 6 chapters. Chapter 1 presents an overview of global antimicrobial resistance (AMR), and reviews the state-of-art strategies to tackle the AMR problems of antibiotic resistance, persisters and biofilms. Chapter 2 summarizes the materials and experimental procedures used in this thesis. Chapter 3 introduces the synthesis and characterization of a novel enantiomeric glycosylated cationic block co-beta-peptides. Chapter 4 discusses its

in vitro antibacterial properties and mechanism of action. With the same co-beta-peptide, Chapter 5 further reveals its activity against antibiotic-tolerant persisters and biofilms with excellent biocompatibility, both *in vitro* and *in vivo*. Chapter 6 presents the conclusions and recommendations for future works.

Section 1.3 Literature review

In this chapter, the major hurdles for effective treatment of infectious disease were reviewed in detail. Mechanisms of antibiotic resistance were discussed (Section 1.3.1). The concept of persister cells and novel strategies to eradicate persister cells were reviewed (Section 1.3.2). Subsequently, biofilms were introduced followed by a detailed review of anti-biofilm strategies (Section 1.3.3).

1.3.1 Antibiotic resistance

The mechanisms of antibiotic resistance have been reviewed below based on the category of antibiotics.

Penicillin, Cephalosporin and other beta-lactam antibiotics

Beta-lactam antibiotics target the enzymes (called penicillin-binding-proteins, PBPs) in cell wall synthesis pathway and inhibit the biosynthesis of cell wall, leading to cell death. The intrinsic resistance could come from an impermeable barrier to prevent entry of antibiotics, as is the case for *P. aeruginosa* due to lack of high-permeable porins on the outer membrane¹⁰. Active efflux pumps serve as another important beta-lactam resistance mechanism for *E. coli*, *P. aeruginosa* and *N. gonorrhoeae*³¹. Beta-lactamase, secreted by many Gram-positive bacteria, could enzymatically digest/hydrolyse beta-lactam antibiotics¹⁰. A notorious pathogen, methicillin-resistant *Staphylococcus aureus* (MRSA), harbours a *mecA* gene to

encode an alternative protein PBP2a that possess cell wall synthesis function whilst having significantly reduced binding affinity to beta-lactam antibiotics³².

Protein and DNA synthesis inhibitors

Bactericidal antibiotic aminoglycosides target the 30S of ribosomal subunit and disrupt the translation and subsequent protein synthesis, leading to cell death. Clinically, the most important resistance mechanism is the enzymatic modification of aminoglycosides, which are commonly mediated by conjugation/transfer of plasmids³³. (Though the outer membrane of Gram-negative bacteria serves as intrinsic barrier of penetration for some aminoglycosides, this mechanism is unimportant clinically.) Ribosomal structure mutation with reduced drug binding affinity is another common resistant mechanism for *E. coli* and *Enterococci*. Since the transport of aminoglycosides across the cytoplasmic membrane is an energy (and transmembrane-potential) dependent process, the efficacy of aminoglycosides is significantly diminished in metabolically inactive bacteria cells such as persisters and small-colony-variants (SCVs)³⁴.

Bacteriostatic antibiotic tetracyclines and macrolides target the 30S and 50S of the ribosomes respectively, inhibiting translation and protein synthesis. Common mechanism of resistance to tetracyclines and macrolides include efflux pump, modified ribosomal structure with reduced affinity to drug and enzymatic degradation of drug³⁵.

Fluoroquinolones target the DNA gyrase or topoisomerase IV to inhibit DNA synthesis. Resistance is commonly acquired by chromosomal gene mutation to modify the target of drug³⁶. Sulfonamide and trimethoprim target the enzymes to synthesize folic acid, a precursor for DNA synthesis. Resistance is primarily

chromosomal or plasmid-mediated mutation to encode an alternative folic acid synthetic pathway with low affinity to drugs³⁷.

Glycopeptides, lipopeptides and polypeptide antibiotics

Colistin competitively displaced the divalent ions that holds the lipopolysaccharide (LPS) together, permeabilize and cross the outer membrane via a “self-promoted uptake”, and interact with the cytoplasmic membrane of Gram-negative bacteria³⁸. Both chromosomal and plasmid-mediated colistin resistance have been reported, which is primarily through modification of LPS to reduce negative charge on the bacterial outer membrane (and hence reduce electrostatic attraction to colistin)³⁹⁻⁴⁰.

Glycopeptides vancomycin and teicoplanin bind to D-Ala-D-Ala residue of the pentapeptide, an ubiquitous component of lipid II, to block cell wall synthesis and leads to cell death⁴¹. Vancomycin resistance was first identified in *Enterococci* and has been reported to transmit to other pathogens such as *S. aureus*. Vancomycin resistance is mediated by a vancomycin-resistant operon (*van*) that encodes D-Ala-D-Lac as alternative building blocks of cell wall with reduced binding affinity to vancomycin⁴². Most commonly identified *van* operons are *vanA*, *vanB* and *vanC*; they are often located at mobile elements (such as transposons). High-level vancomycin-resistant *S. aureus* (VRSA) strains have been isolated from hospitals and their resistance are most commonly mediated by *vanA*⁴³. Besides modification of substrate with reduced binding to vancomycin, “false target” is another mechanism of resistance identified in clinical isolates of vancomycin-intermediate *S. aureus* (VISA). These strains possess reduced activity of PBP4, leading to low cross-linking of the cell wall. This leave behind enriched monomeric mucopeptide with excess D-Ala-D-Ala residues as a “bait” for vancomycin binding to protect

the true target (lipid II)^{41, 44-45}. Moreover, in both lab-generated and clinically isolated VISAs, thickening of cell wall is often observed and believed to function as a “trap” for vancomycin molecules from approaching the actual target⁴⁵⁻⁴⁶.

Lipopeptide daptomycin and polypeptide gramicidin target the cytoplasmic membranes of Gram-positive bacteria. Since antibiotics of this category are positively charged and first interaction with bacteria is electrostatically driven, the mechanisms of resistance are commonly associated with reducing negative charge and/or addition of positive charge through modulation of D-alanylation on cell wall teichoic acid (WTA, anionic component of cell wall) and/or lysylation of phosphatidylglycerol (PG, anionic component of cytoplasmic membrane). Both pathways add positive charge on cell envelope, through the following regulatory genes: (i) *Dlt* operon (*Dlt* A, B, C, D) encodes proteins to add D-alanine (+1 positively charged) to WTA to reduce the overall negative charge of the latter; overexpression of *Dlt* operons promotes D-alanylation⁴⁷; (ii) *FmtA* acts as esterase to remove D-alanine from WTA, hence down-regulation of *FmtA* function reduces esterase activity and retains more D-alanine on WTA⁴⁸; (iii) *MprF* gene encodes protein that both synthesizes the positively charged lipid lysyl-phosphatidylglycerol (LysPG) and translocates LysPG to outer leaflet of the cytoplasmic membrane; gain-of-function mutation in *MprF* gene increases the positive charge on membrane⁴⁹⁻⁵⁰; (iv) Other common pathways includes GraSR, WalKR and VraSR gens, which are stress modulators upon cationic antimicrobial treatments and are commonly referred to as Cationic Antimicrobial Peptide (CAMP) resistance genes. Besides surface charge modulation, thickening of cell wall is also reported in daptomycin-resistant *S. aureus* isolates, which also functions as a “trap” to prevent penetration of daptomycin^{47, 51}. Moreover, cross-

resistance to host defense peptide has been reported due to the similarity of daptomycin and these cationic antimicrobial peptides⁵²⁻⁵³.

Antimicrobial peptides

Antimicrobial peptide (AMP) is the intrinsic arsenal of the innate immune system to eliminate infection. In a post-antibiotic era, AMPs are considered as promising candidates to address the increasing prevalence of antibiotic-resistance. AMPs vary greatly in size (from 5 to 149 amino acids), charge (from -3 to +20), amphiphilicity (hydrophobic moments) and secondary structures (α -helices, β -sheets, relaxed coils). In general, most AMPs are cationic in nature and bear a significant proportion of hydrophobic residues⁵⁴. The most well-accepted AMP mechanism of action is the disruption of bacterial cytoplasmic membrane⁵⁵. Tremendous efforts have been made to study the AMP-membrane interaction and various models have been proposed, from the pore-forming models such as carpet model, Barrel-stave model, toroidal model, to the less lytic models such as anionic lipid clustering model and non-lytic depolarization model⁵⁶. Besides bacterial membrane, AMPs could also target cell wall and intracellular components. The bactericidal activity of human neutrophil protein 1 (hNP-1) and thrombin-induced platelet microbicidal protein 1 (tPMP-1) depends on the presence of cell wall, and cell wall-free protoplasts become resistant to these two AMPs⁵⁷. Wall teichoic acid (WTA)-deficient *S. aureus* showed 10 to 100-fold resistance to Mammalian Group IIA Phospholipase A2 (gIIA PLA2) and Human β -Defensin 3 (hBD-3)⁵⁸, indicating WTA is closely associated in the killing mechanism of these two AMPs. Nisin, a typical lantibiotics, binds to Lipid II as a docking site on cytoplasmic membrane to

facilitate further pore formation/depolarization⁵². Intracellular targets such as nucleic acids and proteins have also been reported as targets for AMPs⁵⁹.

Compared with antibiotics that target essential proteins, bacterial resistance towards AMPs has occurred to a much lesser degree. Nevertheless, AMP resistant bacteria have been isolated both from clinical practice and generated in lab, where common mechanism of AMP resistance are identified as following: (i) Broad CAMP resistance by increasing positive charge on bacterial surface (such as *MprF* gene, *DltABCD* operon, *mcr-1* gene that modulates cytoplasmic membrane, cell wall teichoic acid, Lipid A respectively); (ii) Efflux pumps that actively remove AMPs from cells such as the ABC transporter system for Gram-positive bacteria⁶⁰; (iii) Emergence of Small Colony Variants (SCVs) with either defects in electron transport chain (hemin-/menadione-auxotrophs) or thymidine biosynthesis (thymidine-auxotrophs)³⁴: *S. aureus* SCVs showed reduced susceptibility to AMPs whose killing depend on transmembrane potential $\Delta\psi$ (e.g. platelet microbicidal proteins (PMP-2), hBD-2 and -3), because the $\Delta\psi$ of SCVs are significantly reduced due to defective respiration chain⁶¹⁻⁶². SCVs also showed resistance to AMPs with intracellular targets (e.g. lactoferricin B) due to their low metabolic rate⁶³; (iv) inactivation of AMPs: bacteria can decorate cell surface with negatively charged polysaccharides as a “decoy” for AMPs, such as capsular polysaccharide (CPS) of *S. pneumonia* and polysaccharide intercellular adhesion (PIA) of *S. epidermidis*⁶⁴⁻⁶⁵. Alternatively, bacteria could secrete protease that non-specifically degraded single chain AMPs such as LL37⁶⁶. Microbial resistance to AMPs is a serious concern because cross-resistance among different types of AMPs are commonly identified, and extensive use of AMPs in clinical settings could select for mutants that are resistant to the key components of our own innate immune system⁶⁷.

In summary, the efficacy of antibiotics is severely threatened by the evolution of resistance. According to a high-level study chaired by Sir Jim O'Neil, if the AMR problem is not properly tackled, it is estimated to surpass cancer and become the biggest killer by 2050, resulting in 10 million death per year at a cumulative cost of 100 trillion USD to global economy⁹. To reduce antimicrobial resistance requires global efforts to increase public awareness of AMR, reduce misuse/abuse of antibiotics in agriculture, support global surveillance and promote incentives of investment on new antimicrobial drug development.

Section 1.3.2 Persister cells

I. Definition of persister cells

The phenomenon of persister cells was first observed over 70 years ago by Hobby⁶⁸ and Bigger⁶⁹, where high concentration of bactericidal antibiotic penicillin failed to sterilize the culture of pen-sensitive *S. aureus*. However, the clinical importance of this phenomenon was largely ignored until the last two decades. In recent publications, persister cells are defined as a sub-population of bacteria that can phenotypically survive exposure to a bactericidal antibiotic treatment, yet are genetically identical to their antibiotic-susceptible kin⁷⁰⁻⁷¹. In a Consensus Statement of *Nature Reviews Microbiology* published very recently, the phenomenological definition of antibiotic resistance, tolerance and persistence were presented⁷². A clear cut-off for antibiotic resistance is the increased value of minimal inhibitory concentration (MIC), which is mediated by genetic changes. On the other hand, antibiotic tolerant or persistent cells have the same MIC values as their susceptible kin (Figure 1-1a)⁷³. Tolerance is a generic term to describe the prolonged survival of bacteria cells under antibiotic treatment and is characterized by slower killing.

Tolerance is typically associated with a shift in the MDK₉₉ (minimum duration of treatment that kills 99% of the bacterial population) (Figure 1-1b)⁷². The phenomenon of tolerance has been observed in all major types of clinically relevant pathogens¹⁴. Dormancy is a common mechanism associated with antibiotic tolerance, where reduced metabolism and ATP levels, or even complete growth arrest^{14, 74}. Historically, persister cells are often characterized by biphasic killing of a heterogeneous population, where sensitive cells are killed by antibiotics and persister cells remain survived (Figure 1-1c)¹². The persister cells can resume growth after the antibiotic pressure is removed to produce a progeny population. Because they do not carry a resistance factor genetically, the progeny shows the same susceptibility characteristics to the parental population⁷⁵. Another distinctive feature is that the size of persister is only weakly dependent on the antibiotic concentration, and persister cells are often selected by a concentration far above MIC (e.g. 10 to 100×MIC)^{70, 76}.

Both tolerance and persistence describe a population-level phenomenon. Tolerance refers to the prolonged survival of the population as a whole; persistence is always associated with heterogeneous population, where persister is the antibiotic-tolerant sub-population. When the level of persistence is extremely high in a population (100%), persistence is equivalent to tolerance.

Figure 1-1 Definition of antibiotic (a) resistance, (b) tolerance and (c) persistence.

II. Persister formation

The term “tolerance” and “persistence” are sometimes used inter-changeably in literature¹⁴, partially due to the similar methods to generate these cells. Though the molecular mechanism leading to persister formation is still ambiguous⁷⁷, widely-accepted protocols to generate persisters are listed below. (i) Type I persisters can be triggered by environmental stress factors such as starvation⁷⁸, antibiotic treatment at sub-inhibitory concentration⁷⁹ or cell-cell competition in stationary phase growth⁸⁰. (ii) Type II persisters arise spontaneously in the absence of a “trigger”⁸⁰. For example, *S. aureus* in exponential growth phase could stochastically enter persister state due to a reduced ATP production. This pre-existing sub-population of persister cells can be isolated with antibiotic treatment at $10\times\text{MIC}$ ⁷¹. Stochastic persister formation is believed to be an evolutionary strategy to enable phenotypic heterogeneity in an isogenic population, to maximize the fitness of the whole population in dynamic environments⁸¹. The molecular-level mechanism of both type I and type II persister formation remains elusive, yet at least one well-accepted signalling pathway has been identified, via the stringent response alarmone (p)ppGpp. Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate

(pppGpp) are collectively referred to as (p)ppGpp, which modulates transcriptional reprogramming and direct adjustment of target protein activities⁸². In *E. coli*, the linkage between (p)ppGpp and downstream toxin-antitoxin (TA) modules has been extensively studied as a molecular basis for persister formation. TA system composes of toxins (typically proteins) that inhibit bacterial growth, and antitoxins that impair the function of the corresponding toxins. Overexpressed toxin genes of TA system produces a stable toxin that can disrupt essential cellular activity (e.g. RNA degradation, inactivation of translation factor, diminishing translation activity), and render the bacteria into dormancy state⁷⁷. In one of the many hierarchical signalling pathways of *E. coli* K12, activation of stringent response genes (under the control of (p)ppGpp) produces polyP kinase enzyme, which degrades anti-toxin in TA system and leads to activation of the toxins and persister formation⁸³. Figure 1-2 shows a summary of the pathways that leads to persister formation in *E. coli* (a well-studied model microorganism for persister formation). It is worth noting that all the pathways eventually lead to reduced cellular activity and dormant state.

Figure 1-2 Molecular pathways underlying persistence in *E. coli*.¹¹

The clinical significance of antibiotic persistence has been increasingly recognised in recent years¹¹. Persisters account for roughly half of all infection cases, and are largely responsible for failure of antibiotic treatment, leading to recalcitrant infections^{13, 84}. In fact, clinical isolates from chronic infections (such as *E. coli* from urinary tract infection and *P. aeruginosa* from cystic fibrosis patients) commonly express higher level of persistence¹⁴. Bacteria in persister state is also linked with increased virulence, possibly rising from the stringent response pathways that are activated in persisters¹⁵. Increased level of persistence is also linked to higher evolution of resistance¹⁶. Kim Lewis has defined the two faces of threat towards effective antimicrobial chemotherapy: antibiotic-resistance and persistence/tolerance (Figure 1-3).

Figure 1-3 Antibiotic persistence and tolerance as a significant barrier to effective antimicrobial chemotherapy.⁸⁴

III. Strategies to tackle persisters

Due to the clinical relevance, strategies to eradicate persisters have been proposed, including (i) inhibiting persister formation, (ii) direct killing of persister by membrane-active compounds, (iii) antibiotic target stimulation and (iv) using drug combinations to eradicate persisters.

(i) Inhibiting persister formation

Since bacteria entering persister state shows tolerance to antibiotics, inhibiting the formation of persisters could prolong the efficacy of current antibiotics⁸⁵. HipA (high persistence A) is the first identified toxin in the TA system of *E. coli* that contributes to persistence. A molecule that inhibits HipA has been reported in 2016 to significantly reduce level of persistence in *E. coli*⁸⁶. Similarly, a poly P kinase inhibitor, mesalamine, also reduces the persister formation in *E. coli* and *P. aeruginosa*⁸⁷. Other reported inhibitors of persister formation include respiration inhibitors of stationary phase *E. coli*⁸⁸, and quorum sensing inhibitors which also modulates persister formation⁸⁹.

(ii) Direct killing of persister by membrane-active compounds

Though persisters are not actively dividing, they still need a physically intact membrane to maintain viability. Hurdle *et al* highlighted the potential application of membrane-targeting molecules against persisters⁹⁰. So far, many bacterial membrane-active compounds have been reported, and their activity against persister cells are demonstrated both *in vitro* and *in vivo*⁹¹⁻⁹⁴. Besides membrane lytic, membrane depolarization agents/ionophores that prevent ATP synthesis have been reported to kill persisters⁹⁵⁻⁹⁷. The membrane-targeting compound HT-61 is now in a clinical trial for topical decolonizing the nose of *S. aureus* infections⁹⁶.

Molecules that target essential cellular activity (other than bacterial membranes) have also been reported to kill persisters. Conlon *et al* reported a small molecule (ADEP4) that activates the ClpP protease, causing extensive non-specific protein degradation in non-growing persister MRSA⁷⁰. Mitomycin C, an FDA-approved anti-cancer drug, can be passively transported into both actively dividing and dormant persister cells, leading to spontaneous cross-linking of DNA⁹⁸. Inhibitor of dihydrolipoamide acyltransferase (DlaT) can selectively kill *Mycobacteria tuberculosis* persisters⁹⁹, and inhibitor of demethylmenaquinone methyltransferase (MenG) kills both growing and nutrient-deprived persister cells of *Mycobacterium tuberculosis*¹⁰⁰.

(iii) Sensitizing persister to antibiotics

Since persisters are metabolically inactive and tolerate to antibiotics, strategies to restore the metabolism of persisters could re-sensitize the cells to antibiotics. High-throughput screening identified a small molecule (C10) that could render the persisters to be sensitive to antibiotic norfloxacin and ampicillin⁷⁵. Interestingly, the supernatant of spent culture medium contains resuscitation-

promoting factors (Rpfs) and could resuscitate *S. aureus* persister cells¹⁰¹. Glucose and fumarate (and a suitable terminal electron acceptor) could promote respiratory metabolism in *E. coli*, and sensitize the persisters to quinolones¹⁰². Addition of metabolites such as fructose or pyruvate could stimulate the proton motive force (PMF) to facilitate the uptake of aminoglycoside, and restore the bactericidal effect of the antibiotic gentamicin¹⁰³. Similarly, addition of physiologically compatible amino acid L-arginine makes the medium alkaline, promotes the transmembrane pH gradient (ΔpH) and hence increases the PMF, which leads to re-sensitization of persister cells to aminoglycosides¹⁰⁴.

Besides restoring the energy level of the persister cells, molecules that damages cell membranes could promote influx of antibiotics. For example, silver ion can prime the ROS production and increase membrane permeability of Gram-negative bacteria, and potentiate the activity of antibiotics from various categories against persister cells¹⁰⁵. In another example, a 12-amino-acid cell penetrating peptide was covalently attached to tobramycin, where the peptide permeabilizes the membrane of persister cells to promote uptake of tobramycin¹⁰⁶. A similar design was adopted to produce a vancomycin-d-octaarginine conjugate, where the d-octaarginine peptide could target teichoic acid and vancomycin target the lipid II on membrane, eventually leading to disruption of membrane integrity¹⁰⁷.

(iv) Combination of antibiotics

Membrane-targeting antibiotic colistin, combined with antibiotic with intracellular targets such as gentamicin and ofloxacin, could eradicate the persisters of uropathogenic *E. coli*¹⁰⁸. Combination of daptomycin and tobramycin at high concentrations could eradicate the persister of *S. aureus* which was not achievable by each antibiotic alone⁷⁶. daptomycin and clofazimine in combination showed

enhanced killing against persisters of *B. burgdorferi* generated by amoxicillin treatment¹⁰⁹.

Though various strategies to eradicate persisters have been reported, they suffer from major drawbacks that limit the potential clinical usage. Targeting a single module of the TA system may not be effective to prevent persister formation, due to the redundancy of the TA modulating system and versatile pathways across different pathogens. The combination of antibiotic strategy requires a supra MIC (100×MIC in some case), which leads to potential toxicity issues associated with high dose of antibiotics. Though some molecules could re-sensitize the persister to antibiotics, the practical usage of antibiotic in the abovementioned strategies requires caution, as suboptimal dosing of antibiotics not only leads to resistance development, but also promotes persistence level in the population. The most preferred strategy is the direct killing, regardless of the metabolic inactivity of the persister cells. However, many membrane-active molecules suffer from low selectivity, and their safety profile has not been proven *in vivo*.

Section 1.3.3 Biofilms

I. Definition of biofilm

Besides planktonic phenotype, bacteria also possess a sessile lifestyle called biofilm. Initially misunderstood as a simple “pile” of cells, knowledge of the remarkable physiological and biological complexity has been advanced since the concept of biofilm was proposed in 1980s¹¹⁰. Biofilm is defined as “aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface”¹⁹. The significance of biofilm in infection has been increasingly recognized, whereby US National Institutes of Health has attributed more than 80% of infection

in the body to be biofilm-related²². So far, biofilms have been identified in almost all types of indwelling devices¹¹¹ as shown in Figure 1-4.

Figure 1-4 Biofilm-related infections in human.¹¹¹

The development of biofilm involves the following steps¹¹²: (i) initial adhesion, where the bacteria cells attach to a surface via cell-surface-associated adhesins; (ii) early biofilm formation, where cells produces the EPS and multiply inside the EPS; (iii) maturation, where 3D structure is formed, providing heterogeneous chemical and physical microenvironments for the bacteria residing inside the biofilm; (iv) dispersal, where the bacteria is released from biofilm state to planktonic state¹¹².

II. Extracellular Polymeric Substance (EPS) of biofilm

EPS is the scaffold for the three-dimensional architecture of the biofilm and its function is crucial for the bacteria residing inside. EPS is the physical barrier that

protects bacteria from the harsh external environment. It has been reported to protect bacteria from host immune defence, antimicrobial biocides, antibiotics, toxic metallic cations and even ultraviolet radiation^{17, 20}. This hydrated matrix bridges the bacteria cells to each other, and the whole biofilm to substrate surfaces. It serves as a reservoir of water, ion, carbon source and other nutrients for the bacteria. And the many functions of the EPS matrix are enabled by the vast diverse components in the matrix. Three major components are identified in the EPS, namely polysaccharide, protein, and extracellular DNA (eDNA). Polysaccharide is an indispensable component in the biofilm of many strains, and impaired ability to synthesize polysaccharide significantly diminish the formation of mature biofilm in *E. coli* K12¹¹³. The proteins in EPS can be enzymatic or non-enzymatic (structural). Enzymatic proteins (such as protease, lipase, glucosidase) are secreted by biofilm bacteria to digest biopolymers as carbon sources¹¹⁴. Besides, bacteria also secrete EPS-modifying enzyme as an important strategy to modulate biofilm, which enables biofilm dispersal upon starvation by degrading the EPS. Structural proteins such as extracellular lectins could interact with the polysaccharide in the matrix and stabilize the interaction between bacteria and the EPS¹¹⁵. eDNA is another important component of the EPS, and degradation of eDNA prevented the formation of *P. aeruginosa* biofilm¹¹⁶ and resulted in the degradation of *S. aureus* biofilm¹¹⁷. The eDNA also serves as a niche to disseminate antibiotic-resistant genes via horizontal gene transfer, which is facilitated by the close proximity of a large number of biofilm bacteria¹⁹.

The biofilm components are highly dynamic in response to the external environment. *S. aureus*, a notorious pathogen with versatile pathways for biofilm development, is able to express environmentally regulated biofilms¹¹⁸. When the

environment has high NaCl and low water content (such as on the skin), up-regulation of *ica* locus produces biofilm that major consists of polysaccharide intercellular adhesin (PIA). PIA is a poly- β (1-6)-N-acetylglucosamine (PNAG) and this hydrophilic polymer could help to absorb water to keep the biofilm hydrated¹¹⁹. When the environment is more acidic (such as urinary track, vagina and mouth), *fnb* is up-regulated to promote formation of biofilm that involves fibronectin-binding protein (FnBP). This cell wall anchored (CWA) protein could tightly hold the bacteria cells with the biofilm matrix¹²⁰. In high shear environment with iron- and nutrient deficiency (such as blood), up-regulation of *SaeRS* genes encodes Coagulase to convert the plasma protein fibrinogen to fibrin, which is recruited by bacteria to build the fibrin-type of biofilm¹²¹. Hence, biofilm development is highly dynamic and mediated by the diverse and redundant pathways of bacteria such as *S. aureus* and many other pathogens.

Eradication of biofilm bacteria using conventional antibiotics is challenging, as they may be up to 1,000 times less effective. The tolerance of biofilm bacteria to antibiotics is multi-factorial. First, the EPS may be enriched with compounds that interfere with the activity of antibiotics, such as beta-lactamase accumulated from lysed cells¹²². Second, the EPS serves as a physical barrier to prevent/reduce penetration of antibiotics. Cationic antibiotics such as vancomycin¹²³ and daptomycin¹²¹ could be trapped by the negatively charged components in the EPS, leading to reduced efficacy. However, later studies revealed that many other antibiotics such as tobramycin and ciprofloxacin is not trapped by the EPS and they are able to penetrate deep inside the biofilm matrix; yet they still remain ineffective in eradicating the biofilm bacteria¹¹¹. A third and well-accepted factor is the presence of persister cells inside the biofilm. Biofilm bacteria shows great

heterogeneity in their physiological states, in response to the chemical heterogeneities of their local environment inside biofilm²¹. A visualization of the heterogeneous physiological activity is shown in Figure 1-5, where only the outer layer of cells in *P. aeruginosa* biofilm could produce an alkaline phosphatase in response to phosphate starvation (shown by green fluorescence), and bacteria in the inner layer of biofilm was unable to do so¹²⁴. The diffusion process inside biofilm is not homogenous, and concentration gradients of metabolic substrates (such as nutrients, oxygen) and metabolic wastes are established inside biofilm. For example, oxygen was actively consumed by the outer layer of bacteria and failed to diffuse into deeper layers of biofilm due to the relatively slower diffusion rate compared to the consumption rate²¹. In adaptation to the chemical heterogeneities, bacteria cells near the biofilm-interface could have active metabolism, whilst cells residing deep inside biofilm could enter dormancy state due to the stresses from local environment and become persisters for antibiotics. Moreover, the reduced diffusion rate of antibiotics through the matrix leads to bacterial exposure to subinhibitory concentrations of antibiotics, which is also a trigger for bacteria to enter persistence state (as discussed in Section 1.3.2). These factors lead to significantly reduced efficacy of antibiotic treatment.

Figure 1-5 Heterogeneous physiological activity of *P. aeruginosa* bacteria in biofilm.¹²⁴

III. Strategies to tackle biofilm

The capacity of the bacteria to establish stable biofilms is commonly associated with longer treatment time, frequent relapse and higher resistance propensity. Due to the high clinical relevance of biofilm-related infection and constant failure of standard antibiotic treatment, multiple strategies to tackle biofilm have been developed as discussed below.

(i) Preventing biofilm attachment

The initial step of biofilm formation involves the bacteria attachment to a substrate surface, through weak interactions (such as van der Waals forces) or stronger interactions (such as hydrophobic or electrostatic interactions) via adhesive proteins or lipopolysaccharides¹²⁵. Hence strategies to prevent the attachment are first discussed below.

New surface materials and surface coatings have been invented¹²⁶. Antifouling coatings are developed for various surfaces and implants to prevent the initial attachment of bacteria as well as biofoulant such as proteins. Immobilizing poly(ethylene glycol) (PEG) is one of the most commonly used methods to design antifouling surface coatings. The well-hydrated charge-neutral coating on the surface could retain significant amount of water, forming a preventative layer for bacteria to approach the surface. Similarly, zwitterionic polymers (such as phosphatidylcholine and sulfobetaine) are ultra-hydrophilic and overall charge-neutral, and has also been used as surface coating to prevent biofouling¹²⁷. However, an absolute “zero” attachment of bacteria onto antifouling surfaces is impractical¹²⁸. Hence, bactericidal functionality is a desired feature to prevent colonization and proliferation on the surface. Non-leachable contact-active antimicrobial coatings based on cationic polymer brushes have been developed on catheter surfaces and the

antibiofilm efficacy has been validated in murine catheter-associated urinary tract infection model¹²⁹. Leachable coatings such as antibiotic-releasing¹³⁰ or silver-loaded coatings¹³¹ have also been reported to kill bacteria in the local niche and prevent attachment of bacteria. In clinical practice to prevent biofilm development, the US Centers for Disease Control and Prevention published guidelines for the prevention of central venous catheter (CVC) related infections¹³². Antibiotic lock therapy (ALT) is recommended as a prophylaxis for patients with long-term catheters. Antibiotic at high concentration (100 to 1,000 ×MIC) combined with an anticoagulant is instilled into the lumen of catheters and allowed dwelling for hours to prevent luminal colonization and biofilm formation.

Besides modifying the substrate surface, various strategies are reported to modify the bacteria surface to impair its ability for attachment. An orally active FimH inhibitor can effectively prevent the biofilm formation of uropathogenic *E. coli* (UPEC) in a murine urinary tract infection model. Compared to the mannosylated receptors on the mammalian bladder epithelial cells (which serves as a substrate for bacterial attachment via FimH lectin), the inhibitor has significantly higher binding affinity and competitively binds to FimH lectin on the bacteria, hence blocks the bacteria attachment to mammalian cell surface and prevents biofilm formation¹³³. Some cationic antimicrobial peptides at sub-inhibitory concentrations could also prevent the attachment of bacteria to substrate surface, possibly due to its interference with various pathways to down-regulate biofilm formation¹³⁴. Human cathelicidin LL-37 at sub-inhibitory concentration could down-regulates the quorum sensing systems in *P. aeruginosa*, promote bacteria twitching and surface motility, and reduce attachment onto surface to collectively prevent biofilm formation¹³⁵. Lactoferrin, a ubiquitous iron-chelating AMP found in human and cow milk, could

inhibit biofilm formation of *P. aeruginosa* at sub-MIC concentration. It stimulates twitching and surface motility of the bacteria cells, causing the bacteria to wander across the surface rather than forming cell clusters and biofilms¹³⁶. Human β -defensin 3 (hBD3) down-regulates *ica* operon of *S. aureus* leading to reduced production of PIA and marked attenuation of biofilm formation¹³⁷. Synthetic cationic peptide IDR-1018 inhibited the stringent response alarmone (p)ppGpp, which plays a crucial role for biofilm formation¹³⁸. Halogenated small molecules have also shown inhibition of biofilm formation. Halogenated salicylanilides interfere with mitochondrial metabolism, inhibit filamentation and biofilm formation of *Candida albicans*³. Halogenated furanones down-regulated matrix polysaccharide production, and inhibited the biofilm formation of *Bacillus subtilis*¹³⁹. Halogenated indoles such as 7-benzyloxyindole and 5-iodoindole could also interfered with the genetic pathways to reduce biofilm formation¹⁴⁰⁻¹⁴¹.

(ii) Targeting the EPS matrix

Mechanical disruption: The EPS functions as a protective barrier for biofilm bacteria, hence removal of EPS matrix could facilitate the eradication of biofilm-associated infection. Mechanical disruption and physical removal of biofilm matrix has been a common practice in clinical settings, including surgical-site debridement and irrigation to remove biofilm-associated infections. In dental settings, teeth-brushing is considered an effective measure to remove dental biofilms. Sonication with water sprays could remove 80% of biofilm matrix effectively in 2 minutes¹⁴² and it has been widely adopted in clinics to remove dental plaque and biofilms. Other techniques include interfacial tension (microbubbles)¹⁴² and photodynamic therapy¹⁴³. It is worth noting that the physical removal process could mobilize the live bacteria from biofilm, and these periprosthetic bacteria may spread across the

surface, leading to dissemination of infection. Hence, antimicrobial agents are normally used together or prophylactically with the irrigation process. However, mechanical removal of biofilm is only applicable to the tissues/surfaces that are easily accessible, and applying the techniques to biofilms inside human body is still challenging¹¹².

Passive dispersal: Targeting the chemical components (polysaccharide, protein, and extracellular DNA) is another strategy to remove the EPS. Enzymes degrading the exopolysaccharide have been investigated. Dispersin B, a glycoside hydrolase, hydrolyzes the exopolysaccharide poly- β -1,6-N-acetyl-D-glucosamine (PNAG/PIA) and is shown to degrade the biofilm matrix of various bacteria¹⁴⁴. So far, the company Kane Biotech is developing it into a commercial wound care hydrogel product. Sodium-metaperiodate can oxidize and degrade polysaccharide in the EPS to effectively reduce the biofilm biomass of *S. aureus* biofilm that involves PIA¹⁴⁵. Two glycoside hydrolases, PelA_h and PslG_h, degrades the exopolysaccharides Pel and Psl in the biofilm matrix of *P. aeruginosa*, and their activity has been proven both *in vitro* and *in vivo*¹⁴⁶. Enzymes such as proteinase K target the structural protein components (FnBP A and FnBP B) in EPS, and degrade the biofilm matrix of *S. aureus*¹⁴⁵. eDNA is another important target for enzymatic degradation of EPS, as eDNA is an indispensable structural component and interact with other components (such as polysaccharides, proteins) to contribute to biofilm structural integrity¹⁴⁷. DNase, irrespective of the source of isolation (bacterial or mammalian), could degrade the eDNA in the EPS and induce biofilm matrix disintegration. Dornase alfa, a recombinant human deoxyribonuclease I, has been approved by FDA in 1993 to reduce the sputum viscosity in cystic fibrosis patients. Further studies also proved the biofilm matrix dispersal effect of Dornase alfa in *P. aeruginosa*¹⁴⁸. Since

eDNA is important for structural integrity of EPS and is commonly held in place by DNABII protein, competitive displacement of eDNA from DNABII is an alternative strategy to disintegrate the EPS. A native human monoclonal antibody, TRL1068, has high affinity to DNABII analogues and competitively displace eDNA, leading to biofilm matrix dispersal of *S. aureus* in an *in vivo* murine tissue cage model involving biofilm infection¹⁴⁹. On a separate note, a serine protease Esp isolated from commensal *S. epidermidis* can degrade the serine in the biofilm of pathogenic *S. aureus*, leading to biofilm biomass reduction¹⁵⁰. Other biofilm dispersal effectors include amphipathic biosurfactants secreted by bacteria, which can reduce surface tension to facilitate detachment of biofilm matrix. Phenol-soluble modulins (PSMs) of Gram-positive bacteria¹⁵¹ and rhamnolipids of Gram-negative bacteria¹⁵² are common biosurfactants produced by the biofilm bacteria to modulate the integrity of biofilm matrix. Exogenous addition of biosurfactants could effectively disperse preformed biofilms¹⁵³⁻¹⁵⁴. Following a similar mechanism, synthetic amphipathic surfactants such as cetyltrimethylammonium bromide (CTAB)¹⁵⁵ and sodium dodecyl sulfate (SDS)¹⁵⁶ also showed biofilm dispersal activity. However, the poor hemocompatibility is a common problem associated with (bio)surfactants due to their amphipathic nature – the hydrophobic domain has a strong interaction with the lipid bilayer of erythrocyte membrane, leading to hemolysis¹⁵⁷. In fact, the eukaryote-lytic activity of PSMs is considered as an important virulence factor contributing to the pathogenicity of *S. aureus*¹⁵⁸. In our group, we have designed a biocompatible block copolymer that self-assembles into a nanoparticle with hydrophilic dextran as the corona, which diffuses into preformed biofilm of Gram-positive bacteria and detach the bacteria from biofilm, leading to biofilm dispersal – a process described as nanoscale bacterial debridement¹⁵⁹. The copolymer is non-

toxic in a murine *i.v.* injection model and effectively removed biofilm in a murine wound model.

Though the various enzymes can degrade and disintegrate the biofilm matrix, the stability of enzymes in physiological environment is a challenging problem for potential applications¹⁶⁰. Further, enzymatic/chemical degradation of the EPS could result in a sudden release of live bacteria, hence combination treatment with antibiotics is commonly adopted to eradicate the bacteria and prevent spread of infection.

Active dispersal: Besides passive degradation of EPS matrix, another strategy is to modulate the bacterial cell signalling pathways and activate the biofilm dispersal process. The intracellular level of nucleotide c-di-GMP has a central role in the regulation of biofilm dispersal: an increase in c-di-GMP level promotes biofilm formation and a decrease in c-di-GMP level leads to dispersal¹⁶¹. Expression of plasmid-encoded phosphodiesterase degraded the c-di-GMP in *P. aeruginosa* biofilm and dispersed biofilms formed on a foreign-body located in the peritoneal cavity of mice¹⁶²⁻¹⁶³. Nitric oxide has been reported to regulate the c-di-GMP levels in bacteria cells¹⁶⁴⁻¹⁶⁵. Sub-lethal concentration of nitric oxide stimulates phosphodiesterases activity to degrade c-di-GMP and lead to biofilm dispersal. Sodium nitroprusside (SNP) has been used to generate low level of nitric oxide and exert *P. aeruginosa* biofilm dispersal activity in a *in vivo* foreign-body biofilm infection model¹⁶⁶. Though effective, the stability of SNP and nitric oxide precursors is a potential problem. Hence a stable compound was synthesized to selectively release nitric oxide only upon interaction with biofilm β -lactamase, and it could effectively disperse biofilm of β -lactamases producing *P. aeruginosa*¹⁶⁷. In view of the increased virulence and potential dissemination of

released bacteria from dispersed biofilm¹⁶⁶, a hybrid compound of ciprofloxacin and nitroxide was synthesized, where nitroxide mimics the biofilm dispersal activity of nitric oxide but with improved stability, and ciprofloxacin kills the dispersed bacteria from biofilm¹⁶⁸.

Besides c-di-GMP, bacterial quorum sensing (QS) is also involved in the modulation of bacterial biofilm. The concept of quorum sensing was raised in 1970s, and its relevance to bacterial biofilm has been intensively investigated since late 1980s. Similar to the social behaviours observed in animals (such as ants), a sub-population of bacteria produces metabolically expensive public goods for the community to thrive through communal use of the goods – a phenomenon that was believed under the control of QS. Bacterial QS “involves self-produced extracellular chemical signals, which can accumulate in a local environment to levels that are required to activate transcription of specific genes”¹⁶⁹. A common molecule involved in the QS pathway of Gram-negative bacteria is acylated homoserine lactone (AHL). A synthetic halogenated furanone compound was developed as AHL inhibitor and its biofilm dispersal activity has been demonstrated in *P. aeruginosa* biofilm¹⁷⁰. Besides AHL, other molecules that modulates quorum sensing systems have also been evaluated. Addition of autoinducing peptide (AIP-1) could activate the *agr* pathway to increase expression of protease and decrease expression of surface adhesins, leading to dispersal of MRSA biofilm on orthopedic implant titanium material¹⁷¹. A benzamide-benzimidazole based compound inhibit the *Pseudomonas* quinolone signal (PQS) quorum sensing system, reduced the virulence of *P. aeruginosa* PA14 in murine wound burn and lung infection model¹⁷². Interestingly, natural food extracts have also shown QS inhibition activity, including ajoene (garlic

extract), horseradish juice extract, ginseng extract, honey and green tea extracts^{125, 173-174}. Antibiotics such as azithromycin, ceftazidime and ciprofloxacin are shown as QS inhibitors at sub-inhibitory concentration, and azithromycin treatment has shown improved clinical outcome in cystic fibrosis patients with chronic *P. aeruginosa* infection¹⁷⁵.

Another effector to actively disperse biofilm is the exogenous amino acid. D-amino acid (specifically, a mixture of D-leucine, D-methionine, D-tyrosine and D-tryptophan) are commonly produced by bacteria in stationary phase and it is hypothesized that D-amino acids function as signalling molecules to modulate the biofilm of *B. subtilis*, leads to release of amyloid fibers that linked cells in the biofilm together and eventually biofilm dispersal¹⁷⁶. L-Methionine (L-Met) could induce DNase expression in *P. aeruginosa* to degrade eDNA in EPS, leading to biofilm dispersal. L-Met as adjuvant increased the efficacy of ciprofloxacin treatment in murine chronic lung infection model¹⁷⁷.

A summary of the dispersal strategies is presented in Figure 1-6. Despite passive or active dispersal, biofilm dispersal agents need to be used as adjuvants to antimicrobial agents (such as antibiotics or disinfectants), to minimize dissemination of infection.

Figure 1-6 Summary of strategies to disrupt EPS components in biofilm.¹⁷⁸

(iii) Killing of biofilm bacteria

Since persister cells are largely responsible for the recalcitrance of biofilm against antibiotic treatment, strategies to eradicate persisters could potentially eradicate biofilm-associated bacteria. Indeed many compounds effective against persisters also worked well with biofilm bacteria both *in vitro* and *in vivo*, including small molecules such as ADEP4⁷⁰, retinoid⁹¹ and peptides such as aryl-alkyl-lysines⁹³. In this section, various strategies to eradicate the biofilm bacteria were discussed: targeting iron metabolism in biofilm bacteria; using antibiotic+adjuvants to eradicate biofilm bacteria; antibiofilm peptides/peptidomimetics and polymers.

Targeting iron metabolism in biofilm is an interesting strategy to eradicate biofilm bacteria. Due to the scarcity of iron present in biofilm and the vital function of iron in various signalling and metabolic pathways, the sequester of iron is the “Achilles heel” of biofilm bacteria¹⁷⁹. Interestingly, the transition metal gallium can function as a “Trojan horse” in the iron metabolism of bacteria: bacteria uptake gallium due to its chemical similarity to iron, yet gallium could not exert the functionalities of iron. Hence gallium decreased bacterial iron uptake and interfered with iron signalling pathways, leading to death of both planktonic and biofilm-associated bacteria. The efficacy was demonstrated in murine acute lethal pneumonia and chronic airway biofilm infection¹⁸⁰. Lactoferrin is a peptide with iron chelation property, and its anti-biofilm property has been demonstrated in *P. aeruginosa* biofilms¹⁸¹.

Iron-chelators can also be used as antibiotic adjuvants to eradicate biofilm bacteria. The FDA-approved iron chelators deferoxamine and deferasirox, when used together with tobramycin, could eradicate *P. aeruginosa* bacteria in biofilm established on cystic fibrosis cells¹⁸². Similarly, a clinically used iron chelator (DFP) could potentiate antibiotics against coagulase-negative *Staphylococci* (CNS) biofilm on titanium plates¹⁸³. The various biofilm dispersal compounds (discussed in previous section ii) can also be used as antibiotic adjuvants. Since the persister cells in biofilm are in dormant state, the strategies to eradicate persisters (discussed in Section 1.3.2) are also applicable for biofilm bacteria. Compounds that enhance bacterial metabolism or proton motive force can also be used as adjuvants of antibiotics against biofilm bacteria. Mannitol, L-arginine and metabolites restore the proton motive force and promote uptake of aminoglycosides in persisters, and their adjuvants activity are also verified in biofilm models^{103-104, 184}. Membrane-active

compounds such as silver ion and retinoid enhance the bactericidal effect of antibiotics against biofilm bacteria, and the efficacy has been demonstrated *in vivo*⁹¹.

105 .

Membrane active compounds can not only function as adjuvants, more often they are considered as direct-killing agents against persisters. Indeed, membrane active molecules can also eradicate biofilm bacteria. Synthetic cationic amphiphilic small molecules could eradicate biofilm bacteria via cytoplasmic membrane disruption. L-Lysine based lipidated biphenyls¹⁸⁵, D-Lysine based fatty-acid conjugates¹⁸⁶, quaternary ammonium based amphiphilic small molecules¹⁸⁷ have shown bactericidal efficacy against biofilm bacteria, and their efficacy were verified in murine wound biofilm infection models. The cationic molecules interact with the anionic bacterial membrane via electrostatic interaction, and their hydrophobicity enables interaction with lipid bilayer, leading to significant membrane disruption and bacterial death. Similar mechanism has been proposed for many cationic antimicrobial polymers too, which also showed biofilm bacteria eradication capability¹⁸⁸. Because these molecules target the integrity of bacterial membrane, resistance development is much slower compared to antibiotics that target cellular activity. However, due to the presence of EPS materials, the biofilm bacteria eradication concentration is typically higher than the MIC values of these compounds.

Intriguingly, some AMPs could exert biofilm eradication property at concentrations below MIC. Human cathelicidin LL37, the first identified anti-biofilm peptide by Hancock *et al*, is able to inhibit and disperse preformed *P. aeruginosa* biofilms at as low as 1/16 folds its MIC. Soon afterwards, they developed a library of LL-37 derivatives to explore the antibiofilm property¹⁸⁹. An

optimized antibiofilm peptide, IDR-1018 with only 9 amino acid in length, could not only prevent biofilm formation at sub-inhibitory concentration, but also disperse biofilm biomass (and eradicate biofilm bacteria) of multiple species, including *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, MRSA, *S. typhimurium* and *B. cenocepacia*. The broad-spectrum antibiofilm activity was attributed to its direct interaction and degradation of (p)ppGpp, which is an important signal molecule for stringent response and biofilm modulation. 1018 could penetrate the cytoplasmic membrane of biofilm bacteria and killed them due to inhibition of cell wall biosynthesis and triggering of murine hydrolases¹³⁸. To improve the proteolytic stability, a library of D-amino acid analogues of LL-37 were synthesized and two best compounds DJK-5 and DJK-6 were identified. Similar to 1018, DJK-5 and DJK-6 showed broad spectrum biofilm eradication activity via degradation of (p)ppGpp¹⁹⁰. LL-37 analogues with enhanced cationicity and helicity were developed with improved proteolytic stability, and the lead compound SAAP-148 exhibited biofilm eradication ability both *in vitro* and *in vivo*¹⁹¹. Besides LL-37 and derivatives, AMPs such as SMAP-29¹⁹² and Lactoferrin^{181, 193} have also shown biofilm bacteria eradication activity. A database of biofilm-active antimicrobial peptides (baAMPs) have been collectively created¹⁹⁴ and is accessible at <http://www.baamps.it>

Though the various strategies to eradicate biofilm bacteria is promising, a few important barriers for therapeutic usage need to be addressed. Compared with lab-based well-defined growth condition (such as rich medium or PBS buffer), the real physiological condition in which biofilms are established are far more complicated. The efficacy of antibiofilm compounds needs to be demonstrated in complex environment such as whole blood, blood and serum. Antibiofilm agents

(especially peptide-based compounds) could be significantly affected by protease, high salt concentration, and various macromolecules present in the biofilm environment, and their stability needs to be addressed. Other factors such as possible toxicity towards host cells and production costs need to be considered for future clinical usage.

Section 1.4 Motivation and approach to design a novel co-beta-peptide

Amongst the various synthetic polymer families being explored as peptidomimetics¹⁹⁵⁻¹⁹⁹, beta-peptides are promising because they can exhibit biological activity comparable to natural peptides, but have better proteolytic stability²⁰⁰, and are usually amphiphilic and non-mutagenic²⁰¹. Beta-peptides have been considered for use in diverse therapeutic applications such as antimicrobial agents²⁰²⁻²⁰⁴, vaccine drugs²⁰⁵, protein-protein interaction inhibitors²⁰⁶⁻²⁰⁷, and drug delivery²⁰⁸⁻²⁰⁹. Alpha-peptide antimicrobials are known to form facially amphiphilic (FA) structures that enhance the bactericidal properties but tend to be hemolytic and toxic⁵⁴. Compared to alpha-peptides, beta-peptides have an extra methylene group in the backbone. The hydrophobicity of beta-peptides may be tuned by the structure of the side chains. Further, beta-peptides may be designed to form foldamers exhibiting diverse secondary structures, such as helices and beta-sheets²¹⁰⁻²¹² and complex tertiary and quaternary structures²¹³. Munoz-Guerra and Subirana *et al* reported the first research on nylon-3 and analogues, which included the synthesis and helical propensity of these beta-peptides²¹⁴⁻²¹⁶.

In the development of antimicrobial beta-peptides, previous efforts focus mainly on random co-beta-peptides and optimization of their cationic *versus* hydrophobic beta-lactam residues to reduce hemolysis whilst maintaining good

bactericidal effect²¹⁷⁻²²⁰. There is no reported work on glycosylated block co-beta-peptides. Block co-poly(beta-peptides) are interesting as they may show unique combinations of properties displayed by the individual blocks which are as yet under-exploited for the development of next-generation antibacterials. Also, a strategy for the facile synthesis of block co-beta-peptides has not been previously reported.

In this thesis, we report a simple one-shot one-pot anionic ring opening (co)polymerization (AROP) strategy to synthesize a new series of enantiomeric block co-beta-peptides which cannot be made by sequential copolymerization. Two beta-lactam monomers with contrasting reactivities - a protected D-glucose (**DGu**) beta-lactam and a protected cationic beta-L-lysine (**BLK**) beta-lactam - can be block copolymerized in one-shot. The resulting optimized block co-beta-peptide, **PDGu(7)-block-PBLK(13)**, has interesting antibacterial and anti-biofilm biological properties that are elucidated in detail in the subsequent chapters.

Chapter 2: Experimental materials and procedures

Section 2.1 Materials and equipment

Commercially available materials purchased from Alfa Aesar or Sigma Aldrich were used as received. Daptomycin was purchased from Selleck Chemicals, USA. All other antibiotics were purchased from Sigma Aldrich. 3T3 cell lines were purchased from ATCC. Bacteria strains were purchased from ATCC or kindly provided by BEIresources.org.

Polymer molecular weights were determined by gel permeation chromatography (GPC, Waters) equipped with a 2410 refractive index detector (RID), using two ultrahydrogel columns and sodium acetate buffer (0.5 M NaOAc and 0.5 M AcOH, pH 4.5) as the mobile phase at 40°C with a flow rate of 1.0 mL min⁻¹. Matrix-assisted laser desorption/ionization Time of flight (MALDI-TOF) measurements were obtained using Applied Biosystems 4700 series. Light scattering study were performed with a BI-200SM light scattering system (Brookhaven Instruments). Confocal microscopy was performed with Zeiss LSM 800 system. Super resolution Confocal microscopy was performed on a Leica TCS SP8 STED-3X (Leica Microsystems, Wetzlar, Germany). Field Emission Scanning Electron Microscope (FESEM) images were obtained using Joel JSM-6701F system. Room temperature Transmission Electron Microscopy (TEM) were performed with 120-kV Tecnai 12 microscope (T12, FEI). Cryo-TEM grids were prepared with Vitrobot Mark IV (FEI Company). The grids were imaged on a 300- kV Titan Krios transmission electron microscope (FEI Company). Circular dichroism (CD) spectra were obtained using a Chirascan circular dichroism spectrometer with samples dissolved in buffer in a 1 cm path-length quartz cuvette. Isothermal Titration Calorimetry (ITC) measurements were obtained with PEAQ-ITC MicroCal system (Malvern).

Section 2.2 Synthetic procedures

Monomer synthesis

β^3 -hLys was prepared following a published procedure²²¹. Starting from a commercially available protected form of amino acid L-lysine, β^3 -hLys was obtained in high yield via Arndt-Eistert reaction. Then β^3 -hLys was cyclized following the general procedure of Mukaiyama²²² to generate *N*-Boc- β -lactam-L-hLys. A solution of *N*-Boc- β -lactam-L-hLys (970 mg, 4 mmol) in dichloromethane (5 mL) was added to trifluoroacetic acid (3.1 mL, 40 mmol) dropwise at 0 °C, the mixture was stirred at room temperature for about 1 hour until the *N*-Boc- β -lactam-L-hLys was completely consumed (monitored by TLC). After completion, the mixture was cooled to 0 °C, then quenched with a saturated aqueous solution of NaHCO₃ (20 mL). The reaction was diluted with THF (10 mL) followed by the addition of benzyl chloroformate (0.7 mL, 5 mmol), the resulting mixture was stirred at room temperature for 8 hours and then extracted with diethyl ether (2 $\times$ 20 mL). The combined organic extracts were then dried over anhydrous Na₂SO₄, filtered and

oven-dried 10 mL volumetric flasks. The lactams were dissolved in anhydrous THF and diluted to the mark ($[\text{monomer}] = 0.20 \text{ M}$). The stock solution of 4-*t*-butylbenzoyl chloride (*t*BuBzCl) was prepared by weighing 100.3 mg *t*BuBzCl (0.50 mmol, 98% pure) into a 25 mL volumetric flask and diluting to the mark with THF ($[\textit{tBuBzCl}] = 0.02 \text{ M}$). The stock solution of lithium bis(trimethylsilyl)amide (LiHMDS) was prepared by weighing 215.6 mg LiHMDS (1.25 mmol, 97% pure) into a 25 mL volumetric flask and diluting to the mark with THF ($[\text{LiHMDS}] = 0.05 \text{ M}$).

Into an oven-dried Schlenk tube equipped with a magnetic stir bar was placed a total of 2 mL (0.4 mmol) of monomer solution, adjusted for the desired proportion of each monomer in the polymerization feed (*e.g.*, for **PDGu_p(10)-*b*-PBLK_p(10)**, a 1:1 mixture of **1** and **2**, 1 mL of **1** and 1 mL of **2** stock solutions was used). To the mixture was then added 1 mL (0.02 mmol, 5 mol %) of *t*BuBzCl stock solution. The Schlenk tube was sealed, removed from the glove box and cooled to -30 °C under argon atmosphere. To the stirring reaction solution was then added 1 mL (0.05 mmol, 12.5 mol %) of LiHMDS stock solution. The resulting mixture was stirred at -30 °C for about 8 hours until the reaction finished (monitored by TLC), and was then quenched with methanol. After completion, the solution was transferred into a plastic 50 mL centrifuge tube, and the reaction vial was rinsed with a small amount of THF such that the total tube volume was about 5 mL. Hexane (40 mL) was then added to the tube, from which a white solid precipitated. The mixture was centrifuged, and the supernatant solution was decanted. After two more repetitions of the precipitation/centrifugation procedure, the white pellet was dried overnight

under a nitrogen stream to yield the protected product **PDGu_p(x)-b-PBLK_p(y)** as a white powder. ¹H NMR spectra of **PDGu_p(x)-b-PBLK_p(y)** are shown in Chapter 3.

Debenzylation of Polymers:

Polymer **PDGu_p(10)-b-PBLK_p(10)** (145 mg) and 54 mg (0.48 mmol, approx. 1.2 equiv. to monomers) of potassium *tert*-butoxide (KO*t*-Bu) were dissolved in 5.0 mL of tetrahydrofuran. The polymer solution was added dropwise to a rapidly stirred solution of sodium (160 mg, 7.0 mmol) in liquid ammonia (15 mL) at -78 °C under nitrogen. Sodium was washed in toluene and hexane and cut into small pieces before addition. The reaction mixture was warmed to -55 °C and maintained at this temperature for about 2 hours, after which a saturated aqueous solution of ammonium chloride (NH₄Cl, 10 mL) was added to quench the reaction. Meanwhile, the deep blue color disappeared. The solution was warmed to room temperature in a water bath to evaporate the ammonia. The resulting clear solution was filtered, washed with DI water and dialyzed with 1,000 MWCO tubing for 36 hours with 10 water changes. After lyophilization, **PDGu(7)-b-PBLK(13)** was obtained as an amorphous white solid (>90% yield). Other copolymers (**PDGu(x)-b-PBLK(y)**, x+y=20) were synthesized using the same conditions. NMR integrations showed that the ratio of **DGu** to **BLK** in **PDGu(x)-b-PBLK(y)** differed from the stoichiometric ratio of added monomers **1** and **2** in the polymerization step.

Synthetic procedures of dye-attached polymers

Rhodamine-labeling of Polymers:

Rhodamine B acid chloride was used to label polymers in order to investigate the bacteria killing mechanism. 5 mg polymer was dissolved in 1 ml 0.1 M sodium carbonate/sodium bicarbonate buffer, pH=9. Rhodamine dye, 2 mg/ml Sulforhodamine B acid chloride was prepared in DMF in dark conditions to avoid fluorescence loss and was used immediately. 100 μ L Rhodamine in DMF solution was transferred into 1 mL polymer solution and reacted at room temperature for 1 hour. The excess dye was removed by dialysis using a 1000 Da cut-off dialysis membrane for two days and the water was changed every two hours. The Rhodamine-labeled polymers were obtained by lyophilization, obtaining fluffy solid product with magenta color.

Ruthenocene-labeling of Polymers:

The synthesis of ruthenocene follows the previously reported procedures²²⁴. Ruthenocenecarboxylic acid was first synthesized in two steps starting from ruthenocene via Friedel–Crafts acylation with 2-chlorobenzoyl chloride, followed by cleavage by potassium tertiary butoxide and acidify with HCl. After purification, light yellow color crystals of ruthenocenecarboxylic acid was obtained. Subsequently ruthenocenecarboxylic acid (1g) was dissolved in 20 mL of DCM, followed by addition of 10 mL of oxalyl chloride (7.38 g) and two drops of dry DMF. The reaction was refluxed under nitrogen for 24 hr and solvent was removed under reduced pressure to yield ruthenocenecarbonyl chloride. The as-prepared ruthenocenecarbonyl chloride was used immediately in the next step to conjugate to the co-beta-peptide. 20 mg polymer was dissolved in 1 ml 0.1 M sodium

carbonate/sodium bicarbonate buffer, pH=9. Ruthenocenecarbonyl chloride was dissolved in DMSO at 2 mg/ml, and 100 μ L was transferred into 1 mL polymer solution and reacted at room temperature for 1 hour. The excess dye was removed by dialysis using a 1000 Da cut-off dialysis membrane for two days and the water was changed every two hours. The Ruthenocene-labeled polymers were obtained by lyophilization.

In order to prevent the interference from excess dye attachment in the biological assays, the percentage of dye attachment was kept low (<1%) and the dye signals were not detected in the ^1H NMR of the dye-labeled polymers. Where applicable, the ^1H NMR characterization of the ruthenocene intermediate products are shown in Appendix 1.

MALDI-TOF

The molecular weights of the (a) homocationic homopolymer **PBLK(20)**, (b) homosugar **PDGu(20)** and (c) block copolymer **PDGu(7)-*b*-PBLK(13)** were determined using Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) using α -Cyano-4-hydroxycinnamic acid (CHCA) as the matrix. Polymers were dissolved in DI water at 10 mg/mL. 1 μ L of sample solution was added to the sample plate and air-dried for 1 hour. 1 μ L of CHCA matrix solution (10 mg/mL) was then added to the sample spot and air-dried to allow co-crystallization. Signals were obtained in positive ion mode using Applied Biosystems 4700 series. The mass spectrometry was analyzed using a MATLAB[®] based data analysis workflow with functions provided in Bioinformatics Toolbox[™]. The workflow has been attached as a separate file in Appendix 2.

Dynamic Light Scattering

The testing solution of (a) homocationic **PBLK(20)**, (b) **PBLK(10)** , (c) homosugar **PDGu(20)** and (d) block copolymer **PDGu(7)-*b*-PBLK(13)** were prepared at 1 mg/mL in DI water. Samples were filtered using 0.45 μ m PES filter to remove dust particles. 1mL of the sample solution was transferred to a thoroughly washed glass test tube and the DLS signals were obtained at 45°, 60°, 75°, 90°, 105°, 120°, 135°, 150° using BI-200SM light-scattering system (Brookhaven Instruments). The method of mathematical analysis using the GENDIST package²²⁵ was adopted to calculate the autocorrelation function and hydrodynamic diameter (R_h) based on light scattering follows the protocol published by Schillen *et al.* The gyration diameter (R_g) was calculated based on the published equation²²⁶. The plot x-axis represents $\log(\tau)$, and y-axis represents normalized relaxation time distribution function $\tau A(\tau)$.

Section 2.3 Biological tests

2.3.1. *In vitro* tests

Minimum Inhibitory Concentration (MIC):

Minimum Inhibitory Concentrations, MIC₉₀, were determined by broth microdilution method using 96-well microtiter plates according to CLSI standard protocol with minor modification. A single bacteria colony was stripped out from agar plate and inoculated aerobically in 5 mL Mueller Hinton Broth (Difco[®], Becton, Dickinson and Company) under shaking at 37 °C overnight. The overnight culture was 1:100 diluted in fresh MHB and incubated for 3 hours to reach exponential growth phase. The bacteria were further diluted to 1×10^6 CFU/mL in fresh MHB for MIC test. Polymer was dissolved in Deionized water (DI water) at a concentration

of 10.24 mg/mL and subsequently diluted to 1.024mg/mL in fresh MHB. A series of two-fold dilutions of polymer in MHB was prepared in a 96-well plate (Nunc™, ThermoScientific), achieving a concentration gradient from 512 µg/mL to 1 µg/mL. 50 µL of bacteria in MHB were added to each well of the 96 well plate to get a final volume of 100 µL per well. The plate was incubated aerobically at 37 °C for 18 hours, and the optical density absorbance of each well was measured at a wavelength of 600 nm (TECAN, infinite F200). MIC₉₀ is defined as the lowest concentration that exhibited more than 90% inhibition of the bacteria growth according to the OD₆₀₀ readings. All tests were performed three times independently with two wells for each condition in each test. For tests involving daptomycin, 50 µg/mL CaCl₂ are supplemented to the medium.

The panel of hospital-associated multi-drug resistant MRSA strains representing major lineages of global epidemiology²²⁷ were obtained as described below. Vancomycin-resistant *S. aureus* (Strains #1 to 7) were kindly provided and tested by Prof. Barry Kreiswirth and Dr. José Mediavilla from the Public Health Research Institute, University of Medicine and Dentistry of New Jersey (USA). Daptomycin non-susceptible vancomycin-intermediate MRSA (Strains #8 and 9) were kindly provided by BEIresources.org. Strain #10 was kindly provided by Dr. Adriana Rosato from the Houston Methodist Research Institute (USA). Strains #11 to 13 were kindly provided by Tan Tock Seng Hospital (TTSH, Singapore). Strains #14 to 17 were purchased from ATCC. The characterization information can be retrieved from BEIresources.org or ATCC.org.

Sequence Typing of MRSA strains

Multilocus sequence typing (MLST) characterization for the three VISA strains from local hospital (Strains #11 to 13) were conducted following the protocols

previously reported²²⁸ with minor modifications. Overnight culture from single colony was washed with 10mM Tris buffer, resuspended in 800 µL lysis buffer containing 5mg/mL lysozyme, 10mM EDTA, 10mM Tris. After 1 hour incubation at 37°C with shaking, the suspension was heated to 95°C for 10 min and subsequently transferred to ice. 1 mL ice-cold phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed thoroughly by inverting the tubes for five times, followed by incubation for 5 min on ice. After centrifugation at 20,000g for 20 min, the aqueous layer was transferred to a fresh tube and DNA was precipitated by adding 1mL ice-cold ethanol, followed by incubation for 15 minutes on ice. The DNA pellet was collected by centrifugation and washed once with ice-cold 70% ethanol, and resuspended in 50 µL water. The extracted DNA were amplified by PCR using Novagen KOD Hot Start DNA Polymerase, and the amplified products were sequenced by Sanger sequencing. The obtained sequence was submitted to MLST database (<http://www.mlst.net/>) to obtain the sequence type (ST).

Cytotoxicity assay

Mouse fibroblasts (3T3 cell line) were used for *in vitro* cytotoxicity test. 3T3 cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco™) supplemented with 10% fetal bovine serum (FBS, Gibco™) and Pen-Strep (2 mM glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin, Gibco™). The cells were maintained in a tissue culture flask at 37 °C in a humidified incubator with 5% CO₂ until a monolayer (with greater than 80% confluence) was obtained. Polymer cytotoxicity towards 3T3 fibroblast cells was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in colorimetric assay. Briefly, 3T3 cells were harvested from the confluence flask by trypsinization.

Cell number was determined using a hemocytometer and the cell concentration was adjusted to 10^5 cells/mL. 2×10^4 cells per well (200 μ L cell suspension) was added into the wells of a 96-well tissue culture plate and incubated at 37 °C in a humidified incubator with 5% CO₂ for 24 hours without agitation. Polymer stock solution was prepared in PBS (phosphate buffer saline, Gibco™) at a concentration of 10 mg/mL and diluted to desired concentrations in DMEM complete medium. Polymer in DMEM solution was added into the cell seeded 96-well plate. Cells with only DMEM were used as positive controls. The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 24 hours without agitation. The culture medium was subsequently discarded, and the cells were rinsed with PBS. MTT (stock solution of 5mg/ml in PBS) was diluted in DMEM to a concentration of 1mg/mL and was added into each well. The plate was incubated in a CO₂ incubator for 4 hours, after which the MTT solution was aspirated and 100 μ L dimethyl sulfoxide (DMSO) was added into each well. After mild shaking at 150 rpm for 10 minutes, the absorbance of each well was measured at 570 nm using a microplate reader spectrophotometer (BIO-RAD, Benchmark Plus). The percentage cell viability was calculated using the following formula:

$$\% \text{ Cell viability} = \frac{\text{Average abs of treated cells}}{\text{Average abs of controls}} \times 100\%$$

Hemolysis Assay

Fresh human blood was collected from a healthy donor (age 23, Male, IRB-2015-03-040). 1 mL blood was mixed with 9mL PBS and centrifuged at 1,000 rpm for 5 min. The red blood cell pellet was collected and subsequently washed with PBS three times and resuspended in PBS to a final concentration of 5% v/v. Polymer was

dissolved in PBS at 40,000 µg/mL and two-fold serial diluted in a 96-well microplate (Nunc™, ThermoScientific). 50 µL red blood cell in PBS suspension was mixed with 50 µL polymer solution in each well and incubated for 1 hour at 37 °C under mild shaking. The microplate was centrifuged at 1,000 rpm for 10 min. 80 µL aliquots of the supernatant were then transferred to a new 96-well microplate and diluted with another 80 µL of PBS. Hemolytic activity was calculated by measuring absorbance at 540 nm using a 96-well plate spectrophotometer (Benchmark Plus, BIO-RAD). Triton X-100 (0.1% in PBS), which lysed red blood cells completely, was used as positive control, while PBS was used as negative control. The hemolysis percentage was calculated using the following formula:

$$\text{Hemolysis\%} = \frac{O_p - O_b}{O_t - O_b} \times 100\%$$

where O_p is the absorbance of polymer, O_b is the absorbance of negative control and O_t is the absorbance of positive control. All tests were done in triplicate and HC_{10} values were interpolated using mean values of triplicate measurements.

Time kill assay

Time kill study was conducted by incubating bacteria with different concentrations of polymer/antibiotics and determining CFU/mL at various time points. For replicating bacteria time kill study, 10^6 CFU/mL MRSA USA300 in MHB were prepared as previously described. Polymer and antibiotic were added to 1 mL bacteria in MHB suspension in Eppendorf tubes to achieve a final polymer/antibiotic concentration of 4×MIC, 2×MIC, 1×MIC and 0.5×MIC. Bacteria in MHB suspension without addition of polymer or antibiotic were used as positive control. The Eppendorf tubes were incubated aerobically under shaking at 37 °C. At $t = 0$

hour, 0.5 hour, 1 hour, 2 hours, 4 hours, 5 hours, 7 hours and 24 hours, 20 μ L of each sample was extracted and ten-fold serial diluted in PBS for plating onto agar plates. CFU/mL of each sample was determined after 20 hours incubation. Error bars were produced from two independent tests, with two study samples in each test.

Resistance evolution assays

Spontaneous mutation frequency

At day 1, initial inoculum of 3.5×10^9 CFU exponential phase MRSA USA300 in 10mL MHB were placed in 50mL falcon tubes and challenged with polymer at $10 \times \text{MIC}$ under shaking at 37°C . Polymer was changed every 48 hours during the incubation. The $\text{OD}_{600\text{nm}}$ values were recorded daily over 6 days. At days 3 and 6, 100 μ L sample were serially diluted in PBS and plated on nutrient agar plates for CFU determination.

Resistance development by serial passage

Exponential phase MRSA USA300/BAA38/BAA40 cells (10^6 CFU) were grown in 1 mL of MHB containing copolymer at different concentrations: $0.25 \times \text{MIC}$, $0.5 \times \text{MIC}$, $1 \times \text{MIC}$, $2 \times \text{MIC}$ and $4 \times \text{MIC}$. At 24 hour intervals, the cultures were checked for growth. The MIC value for each day was recorded. Cultures from the second highest concentrations that allowed growth ($\text{OD}_{600} \geq 1$) were diluted 1:1000 into fresh media containing $0.25 \times \text{MIC}$, $0.5 \times \text{MIC}$, $1 \times \text{MIC}$, $2 \times \text{MIC}$ and $4 \times \text{MIC}$ of copolymer/ciprofloxacin. This serial passaging was repeated daily for 14 days. Three independent biological replicates were conducted for each experiment. Ciprofloxacin was used as antibiotic control.

Microscopic studies

Confocal Microscopy using Stimulated Emission Depletion Microscopy (STED)

To prepare samples for super resolution STED microscopy, bacteria were taken from agar plate stock and incubated overnight. 5 μ L of overnight bacteria suspension was inoculated in 5 mL fresh MHB medium and incubated for 3 hours to reach log phase. Bacteria cells were then pelleted by centrifugation at 3,000 rpm for 10 min, suspended in culture media at a concentration of 10^8 CFU/mL and incubated for 1 hour in the dark with rhodamine-labelled polymer. Membrane stain FM1-43FX (5 μ g/ml; Life Technologies) was added to the samples before washing the cells three times with PBS and resuspending in a fixative solution of 2% paraformaldehyde in PBS (pH 7.0). Cells were fixed for 2 hours at room temperature, washed three times in PBS and applied to a sterile glass bottom collagen coated dish (MatTek Corporation). STED super resolution microscopy was performed on a Leica TCS SP8 STED-3X (Leica Microsystems, Wetzlar, Germany) at SingHealth Advanced Biomaging Core. Image deconvolution was done using Huygens Professional software (Scientific Volume Imaging, Hilversum, Netherlands). Polymer was covalently labelled with Rhodamine b.

Cryo-TEM microscopy

Log phase bacteria (MRSA USA300) were prepared as previously described. The bacteria were diluted to 10^8 CFU/mL in PBS and incubated with polymer at 37 °C for 4 hours. Bacteria suspension was centrifuged and resuspended in PBS to prepare samples for cryo-TEM at National University Singapore Center for Bioimaging Science. 4 μ L bacteria culture was applied on a Quatafoil 2/2 grids and frozen at liquid nitrogen temperature using a Vitrobot Mark IV (FEI Company). The grids were imaged on a 300- kV Titan Krios transmission electron microscope (FEI

Company) at liquid nitrogen temperature. Images were collected with a nominal magnification of 18000 $\times$ and a pixel size of 4.6 angstrom.

Membrane assays

Propidium iodide staining

Propidium iodide (L13152 Invitrogen) was used following the manufacturers' protocol. Log phase MRSA USA300 was grown as previously described and washed three times with PBS, and resuspended to a final concentration of 10^8 CFU/mL in PBS. Polymers were added to bacteria suspension to achieve desired concentrations and incubated for 30 minutes or 1.5 hours before washing and staining with PI dye for 15 minutes. Samples were washed twice, diluted to 10^7 CFU/mL in PBS and analyzed using Flow cytometry (BD Accuri C6 plus). Data were plotted as histogram of FL3-A channel. Bacteria without polymer treatment, dead bacteria (70°C, 30min) and nisin treated bacteria (128 μ g/mL) served as negative control, positive control and antibiotics control respectively. Data gating and analysis was done using FCS Express 6 plus.

DiSC₃5 depolarization

Cytoplasmic membrane depolarization of the polymer was determined using the membrane potential-sensitive dye DiSC₃5. Bacteria cells in mid-log phase were centrifuged and washed twice using 5mM HEPES buffer (pH 7.8) containing 20mM Glucose and 0.1M KCl. The bacteria were resuspended in the same buffer and diluted to a final concentration of 10^7 CFU/mL. DiSC₃5 solution was added to bacteria suspension to achieve a final concentration of 100nM. DiSC₃5 dye was gradually quenched at room temperature for 30 minutes before added to a black 96

well plate (Costar). Polymer solution was added to achieve desired concentration in the 96 well plate. Gramicidin S (8 $\mu\text{g/mL}$) was used as positive control. Changes in fluorescence due to the disruption of the membrane potential gradient ($\Delta\psi$) across the cytoplasmic membrane were recorded 5 minutes after polymer addition with Tecan reader at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Errors bar were produced from triplicate measurements. Data are presented as folds fluorescent intensity change vs untreated bacteria control. Gramicidin S control resulted in 8.5 folds change of intensity.

Preparation of model liposome systems

Liposomes were prepared by dissolving POPG (2-oleoyl-1-palmitoyl-sn-glycero-3-phospho-rac-(1-glycerol), Avanti company) or POPC (2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine, Avanti company) in chloroform at 10 mg/mL and vacuum drying at room temperature for 30 minutes. The lipid thin film was rehydrated with DI water, sonicated in hot water bath for 2 minutes, followed by vigorous vortexing for 2 minutes. The sonication-vortexing process was repeated 3 more times and the lipid suspension was extruded manually using a Mini-Extruder (Avanti[®]) through a 100nm membrane to obtain unilamellar liposomes.

Secondary structure study

For secondary structure study, polymer was dissolved at 0.05 mg/mL in DI water, 10 mM phosphate buffer (pH 2.6-8.7), 20 mM carbonate buffer (pH 10.8) and in the presence of POPG liposomes (model bacterial membrane) and POPC liposomes (model mammalian membrane). Circular dichroism (CD) spectra were obtained using a Chirascan circular dichroism spectrometer with samples dissolved in buffer in a 1 cm path-length quartz cuvette. Ellipticities θ of each sample were measured

from 190 nm to 260 nm with 0.5 nm step size, with each measurement performed twice. The final data are presented as the mean value after background extraction. Molar ellipticity $[\theta]$ was calculated based on the molecular weight of each polymer measured by MALDI-TOF using the following equation:

$$[\theta] = \frac{100 \times \theta}{C \times l}$$

where θ is ellipticity measured by CD spectrometer in mdeg, C is molar concentration of the compound, l is the path length in cm.

Isothermal Titration Calorimetry (ITC) measurements

The ITC data were obtained with PEAQ-ITC MicroCal system (Malvern). Polymer and liposome solution were prepared in 10mM Tris buffer (with 100 mM NaCl, pH 7.4). 0.15mM copolymer was placed in syringe, and 5mM liposome solution was placed in cell (250 μ L). A total of 30 injections were performed, with each injection of 1.5 μ L copolymer solution into the POPG or POPC in cell. 150s interval between each injection was adopted. The reference power was set at 10 μ W and temperature was set at 37°C. The heat of dilution (baseline) was conducted by injecting copolymer (in syringe) into buffer (in cell) under the same setting. For each sample, baseline was subtracted to calculate heat of reaction and thermodynamic parameters. Data were processed and plotted using MicroCal Analysis Software (Malvern).

TEM embedding, sectioning and imaging

LAC* or LAC* Δ tagO log phase bacteria at 10^8 CFU/mL in PBS were treated with ruthenocene-labelled co-beta-peptide at $1 \times \text{MIC}$ for 4 hours. Bacteria cells were washed with PBS twice, fixed in 2% glutaraldehyde at 4°C overnight. Cells were

then washed twice with DI water, and incubated with 2% uranyl acetate for 5 min. Samples were then washed twice with DI water and stained with 2% osmium tetroxide. Samples were then dehydrated by a series of acetone solution (50%, 70%, 90%, and 100% acetone respectively). Cells were subsequently embedded in Araldite resin. The embedded samples were trimmed, cut to 50 nm ultrathin sections and mounted on 75-mesh thin bar copper grids. The grids were imaged by FEI Tecnai 12 microscope (T12, 120 kV).

Synergistic study

The checkerboard method was used to assess the synergistic activity of antibiotic oxacillin and co-beta-peptide against bacteria. The MICs of each compound were determined as described above. For synergistic study, serial 2-fold dilutions of oxacillin (starting from $16\times\text{MIC}$) and polymer (starting from $8\times\text{MIC}$) were prepared in MHB in a 2 mL deep 96-well plate. After dilution, 25 μL of antibiotic in MHB (along y-axis) and 25 μL of polymer in MHB (along x-axis) was added into each well of a fresh 96-well plate (Nunc). Log phase bacteria at a concentration of 10^6 CFU/mL in MHB was prepared as described above. 50 μL of bacterial inoculum was added into each well to make a final volume of 100 μL . The plate was mixed thoroughly by thermomixer at 1000 rpm for 30s. The plate was then incubated at 37°C for 16-18 hours under aerobic conditions. The bacterial growth was examined by measuring the optical density at a wavelength of 600nm (TECAN, infinite F200). The fractional inhibition concentration (FICs) were calculated as follows: $\Sigma\text{FIC} = \text{FIC}_A + \text{FIC}_B$, where FIC_A is the ratio of MIC of A in the combination/MIC of A alone, and FIC_B is the ratio of MIC of B in the combination/MIC of B alone. A representative setup of synergy plate was shown below.

Kill kinetics of non-replicating (nutrient-starved), stationary phase and antibiotic-generated persisters

A culture of MRSA USA300 or SA29213 was washed two times with PBS and resuspended in PBS at a final concentration of 10^8 CFU/mL. The bacteria suspension was incubated in PBS for 1 hour to adapt the cells to starvation. Polymer and antibiotic were added to 1 mL bacteria in PBS suspension in Eppendorf tubes to achieve a desired final polymer/antibiotic concentration. The Eppendorf tubes were incubated aerobically under shaking at 37 °C. At desired time points, 20 μ L of each sample was serially diluted in PBS, and plated on nutrient agar plates for CFU determination.

For killing of stationary phase bacteria, the overnight culture of MRSA USA300 or SA29213 was directly used without further dilution. Copolymer or antibiotic controls at desired concentration were added to 1 mL stationary phase culture in Eppendorf tubes and incubated aerobically under shaking at 37 °C. At desired time

points, 20 μ L of each sample was serial diluted in PBS, and plated on nutrient agar plates for CFU determination.

For killing of persister bacteria that escaped standard antibiotic treatment, 10^8 CFU log phase bacteria (MRSA USA300 or SA292913) in 1mL MHB were challenged with antibiotics (ciprofloxacin or gentamicin) at $10\times$ MIC for 18 hours. Half of the bacteria were washed to remove antibiotics and challenged with copolymer at $4\times$ MIC in MHB. The other half continued under challenge with antibiotics as a control. Aliquots of samples at each time point were washed with PBS twice to remove antibiotics/polymers and serial diluted in PBS to determine CFU. Error bars were produced from two independent tests, with duplicate samples for each test.

Pre-formed biofilm assays of MRSA and MRSE

In vitro biofilm tests were performed using the MBEC™ assay (Innovotech, Canada) following the manufacturer's protocol. For biofilm involving Fibronectin-binding proteins, 150 μ L of MRSA USA300 bacteria in tryptic soy broth (TSB) containing 1% glucose (initial inoculum of 10^6 CFU/well) was added into each well of an MBEC plate. After 24 hours incubation at 37 °C under 110 rpm mild shaking, biofilm was established on the pegs of the MBEC microtiter plate. The pegs were washed twice using 200 μ L PBS before transfer to the challenge plate. The challenge plate was prepared in a 96-well plate containing a two-fold dilution series of polymer in PBS (200 μ L/well). MBEC pegs were exposed to the challenge plate for 3.5 hours under shaking. Afterwards the MBEC pegs were washed twice with PBS and transferred to the recovery plate. The recovery plate was prepared in a 96-well plate with MHB containing 0.5% Tween 20 solution (200 μ L/well). The recovery plate was sonicated on ice for 30 minutes using ultrasonic bath to fully disintegrate

biofilm and release viable bacteria into the recovery plate wells. The contents of each recovery plate well were 10-folds diluted in PBS and plated on agar plates to determine the bacteria CFU on each peg. The tests were performed three times independently, with four replicates in each independent test.

The plate setup to determine minimum biofilm eradication concentration (MBEC) is illustrated below.

For FESEM imaging of pegs, untreated control and copolymer treated (64 $\mu\text{g/mL}$ for 3.5 hours) pegs were removed aseptically, fixed with 4% paraformaldehyde at 4°C overnight and dehydrated using a graded ethanol series. The pegs were dried under vacuum before imaging with FESEM (Joel JSM-6701F).

To study the interaction of copolymer and homocationic polymer with biofilms using confocal microscopy, 24-hour preformed MRSA USA300 biofilm was established in collagen coated glass bottom petri dish (MatTek). Biofilm was stained with *BacLight*TM live/dead kit under manufacturer recommended conditions for 15 minutes. Polymer in PBS solution (32 $\mu\text{g/mL}$) was dropwise added to avoid physical disturbances to biofilm and confocal images were taken immediately after polymer addition and at defined times thereafter.

To investigate the viability of dispersed biofilm bacteria after copolymer treatment, the supernatant of 32µg/mL copolymer treated biofilm was gently taken from the petri dish and added to a new collagen coated glass bottom petri dish. The bacteria were stained with *BacLight*TM live/dead kit for 15 minutes before confocal microscopy imaging.

To further investigate biofilm formed with polysaccharide intercellular adhesin, various strains of HA-MRSA and MRSE biofilms were established under high salt conditions promoting *ica* locus expression. The MBEC biofilm establishment procedures largely remain the same as previously described, except that TSB + 4% NaCl was used as the biofilm-growth medium. The 24-hour preformed biofilms were challenged with copolymer or vancomycin antibiotic challenge solutions before determining viable bacteria CFU on each peg.

Biofilm dispersal by crystal violet staining

Overnight culture of Gram-positive bacteria were diluted to 10⁶ CFU/mL in TSB with 1% glucose (Tryptic Soy Broth, BD). For *S. mutans*, TSB + 1% sucrose was used. 180 µL of bacteria suspension were added to 96 well plates and incubated for 24 to 48 hours at 37°C statically. Visible biofilms were established at the bottom of each well. The pre-formed biofilms were gently washed with DI water twice, and challenged with 2-fold serial diluted compound solution (co-beta-peptide or antibiotic/antiseptic control) for 4 hours. The compound solution was gently discarded and the biofilms were gently washed with DI water twice. 1% crystal violet solution were added to stain the biofilm residue in the wells for 15 minutes. The crystal violet solution was discarded and the plate was washed twice with DI

water to remove excess staining solution. 30% acetic acid was added to dissolve the crystal violet stains. The biofilm biomass was quantified by measuring the optical density at a wavelength of 600nm (TECAN, infinite F200). Both untreated control (bacteria only) and sterile control (MHB only) were included. Each compound was tested in four replicates.

2.3.2. *In vivo* tests

***In vivo* Murine Systemic Toxicity (Intravenous Injection) Model:**

The animal experiments were reviewed and approved by the Animal Ethics and Welfare Committee (AEWC) of Ningbo University. Female Balb/c mice (6-7 weeks, 18-22 g) were acclimated to the animal facility for at least 48 hours prior to experimentation. *In vivo* toxicity test was conducted for **PDGu(7)-b-PBLK(13)** in a murine intravenous injection model. Compounds were injected intravenously at a concentration of 10 mg/kg body weight in 200 μ L PBS daily for 7 consecutive days. Each group consisted of five mice and their condition was continuously monitored for a period of 14 days. All mice survived until day 7 after the final **PDGu(7)-b-PBLK(13)** injection. Clinically significant biomarkers were recorded before, and 24 hours, 4 days and 7 days after the first injection using Blood Chemistry Analyzer Pointcare V2 (MNChip). The data are expressed as mean $\pm$ standard deviation. Alanine transaminase (ALT) level, aspartate transaminase (AST) level and their ratio (AST/ALT) are clinically significant biomarkers for hepatotoxicity. AST and ALT are enzymes normally contained in liver cells. Upon liver damage, the enzymes are released into blood and an increase of the enzyme level can be directly related to the extent of tissue damage²²⁹. TBIL (total bilirubin) measures the total amount of bilirubin (both conjugated and unconjugated with sugar) in the serum. Bilirubin

is a catalytic product during the destruction of aged red blood cells, and it is processed by the liver for its elimination from the body. Possible causes of high TBIL include liver damage and elevated red blood cell hemolysis²³⁰. Serum creatinine (CRE) is a muscle metabolic byproduct and is cleared in the kidney. Elevated creatinine level is an indicator of kidney damage that leads to reduced clearance of creatinine. Blood glucose (GLU), blood urea nitrogen (BUN), potassium and sodium ion concentrations are other common biomarkers for kidney function and the electrolyte balance in the blood. p values were calculated using one-way ANOVA followed by Dunnett multiple comparison test.

For histopathology studies, mice were sacrificed at 48 hours post last injection, and histological analysis was performed on the tissues obtained from their harvested organs (liver, kidney, and spleen). Tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The sections were examined by a pathologist and no pathological changes were detected in comparison of tissue sections of treated mice with that of the controls. These results provide evidence that the prepared formulation has negligible acute tissue toxicity.

***In vivo* infection models**

The animal experiments were reviewed and approved by the Animal Ethics and Welfare Committee (AEWC) of Ningbo University and the Nanyang Technological University Institutional Animal Care and Use Committee (NTU-IACUC).

Acute infection models

For acute wound infection model, female C57BL6 mice aged 8 weeks were used for excision wound model to evaluate *in vivo* antimicrobial efficacy of polymer. All mice were housed on a 12 hour light-dark cycle at room temperature for one week

prior to the experiment. Mice were anesthetized using isoflurane and hair from the back was removed with a shaver and sterile scalpel blade. The shaved area was further sterilized using 70% ethanol and a 5 mm diameter excision wound was created using a biopsy punch. 2.5 μ L MRSA USA300 in PBS suspension (5×10^5 CFU/mL) was added to the wound site and covered by Tegaderm (3MTM) to protect from contamination. 4-hour post infection, mice were anesthetized, PBS vehicle, polymer or vancomycin at desired concentration were applied to the wound sites. The wound sites were covered by Tegaderm again. Tissue samples were harvested 24 hours after infection, homogenized in 1mL PBS, serial-diluted and plated onto agar plates to determine bacteria CFU.

For acute systemic infection model, eight-week-old female balb/c mice were i.p. infected with 10^8 CFU/mL of stationary-phase MRSA USA300 suspended in 200 μ l of PBS (with 5% mucin). Infections were allowed to develop for 2 hours before initiation of treatment. Vehicle alone, copolymer or vancomycin was injected i.p. at 5 mg/kg in 200 μ L PBS. 24 hour post infection, mice were sacrificed and kidney and spleen were harvested and homogenized to determine CFU. IP fluid was harvested by flushing the abdominal cavity with 2mL PBS and subsequent recovery of fluids (>1mL) after massaging the abdomen. For survival test, mice were infected and treated as previously described and monitored up to 96 hours post infection/treatment.

Established (biofilm) infection models

For deep-seated thigh infection model, six-week-old female CD1 ICR mice (20-25 g) were rendered neutropenic by cyclophosphamide injection. 150 mg/kg and 100 mg/kg of cyclophosphamide were administered via i.p. injection at 4 days and 1 day

before infection, respectively. On the day of infection, 10^5 cells of stationary-phase MRSA USA300 suspended in 50 μ l of saline were injected to the thigh of each mouse using 1mL insulin syringe. At 24 h post-infection, mice thigh tissues were severely infected. Mice were treated with vehicle alone, 20 mg/kg copolymer or antibiotic subcutaneously; 3 hours later, 2nd treatments were given by the same route. Mice thigh tissues were harvested 24 hours post first treatment and homogenized to determine bacteria CFU.

For t = 24 hour wound biofilm model, single excisional wound was created on the dorsal area of female C57BL6 mice aged 8 weeks (n = 6 per group). Bacteria was inoculated as described above, and Tegaderm was used to cover the wound site. The infection was allowed to develop for 72 hours before initiation of treatment. Six treatments over 2 days (three treatments, four hours apart, per day), were applied. Tissue samples were harvested 4 hours after the last treatment and homogenized to determine bacteria CFU.

For t = 72 hour wound biofilm model, single excisional wound was created on the dorsal area of female C57BL6 mice aged 8 weeks (n = 6 per group). Bacteria was inoculated as described above, and Tegaderm was used to cover the wound site. The infection was allowed to develop for 72 hours before initiation of treatment. Six treatments over 2 days (three treatments, four hours apart, per day), were applied. Tissue samples were harvested 4 hours after the last treatment and homogenized to determine bacteria CFU.

***Ex vivo* wounded human skin biofilm model**

Human skin samples were purchased from Biopredic International. All samples were obtained from healthy donors undergoing cosmetic surgery and informed

consent was given in accordance with French law and ethical principles. 5mm diameter wounds were created and inoculated with 10 μ L MRSA USA300 (2×10^9 CFU/mL). Infections were developed for 48 hours and wound sites were gently rinsed with PBS to remove planktonic bacteria; PBS vehicle alone, 100 μ g vancomycin or copolymer were applied three times with a 3-hour interval between each treatment. 3 hours post last treatment, samples were harvested and homogenized for CFU determination.

Chapter 3 Synthesis and characterization of the enantiomeric glycosylated cationic block co(beta-peptides)

In this Chapter, we report a simple one-shot one-pot anionic ring opening (co)polymerization (AROP) strategy to synthesize a new series of enantiomeric block co-beta-peptides which cannot be made by sequential copolymerization. Two beta-lactam monomers with contrasting reactivities - a protected D-glucose (**DGu**) beta-lactam and a protected cationic beta-L-lysine (**BLK**) beta-lactam - can be block copolymerized in one-shot.

Section 3.1 Synthesis of the (co)polymers via one-shot one-pot AROP

The monomers *N*-Cbz- β -lactam-L-lysine (**BLK_p**) and *O*-Bn- β -lactam-D-glucose (**DGu_p**) were synthesized and verified by Nuclear Magnetic Resonance (NMR) spectroscopy (Figure 3-1).

Figure 3-1 NMR spectra of **BLK_p**. (a) ¹H & (b) ¹³C

The synthetic strategy relies on the observation that the homopolymerization of *N*-Cbz- β -lactam-L-lysine monomer (**BLK_p**) is much slower than the homopolymerization of *O*-Bn- β -lactam-D-glucose monomer (**DGu_p**) (Table 3-1).

Table 3-1 Screening of monomers via relative rates of homopolymerization^a

Monomer	Reaction time for homopolymerization ^a
<i>N</i> -Cbz- β -lactam-L-hLys (BLK_p)	8 hours ^b
<i>N</i> -Cbz- β -lactam-L-hOrnithine	6 hours
<i>N</i> -Cbz-MM β 220	2 hours
O-Bn- β -lactam-D-glucose (DGu_p) 231	10 min
O-Bn- β -lactam-D-galactose 232	10 min

^a Approximate reaction time for complete homopolymerization performed at $-30\text{ }^{\circ}\text{C}$. Theoretical degree of polymerization ($\text{DP}_{\text{theo}} = 20$). ^b The well-controlled AROP synthesis of poly-beta-L-hLys (**PBLK**) has not been reported previously. Room temperature polymerization leads to the reaction finishing immediately after initiation by base, which also yielded the protected polymer **PBLK_p** with a higher molecular weight than anticipated based on stoichiometry. The initiation rate of **BLK_p** appears to be much slower than its rate of propagation, which is undesirable. Reducing the reaction temperature reduces both initiation and propagation rates. When AROP was carried out at $-30\text{ }^{\circ}\text{C}$, the degree of polymerization of **PBLK_p(20)** ($\text{DP}_{\text{theo}} = 20$) matched the stoichiometric value. Complete homopolymerization of the monomer **BLK_p** requires about 8 hours at $-30\text{ }^{\circ}\text{C}$, while the monomer is essentially quantitatively recovered if reacted for only 10 minutes. After deprotection by sodium in ammonia, the molecular weight of the water-soluble **PBLK(20)** calculated based by using gel permeation chromatography (GPC) was close to the theoretical value with an acceptable dispersity ($D = 1.2$).

When **DGu_p** and **BLK_p** monomers (10:10, mole/mole) were mixed together in tetrahydrofuran, **DGu_p** was totally consumed in 8 minutes while the **BLK_p** monomer required 8 hours for complete reaction (Figure 3-2 a-c). The molecular weight of the product increased linearly over an 8-hour period during which **DGu_p** disappeared rapidly in the first few minutes, while **BLK_p** was consumed gradually over the next few hours (Figure 3-2d). A plot of molecular weight (M_n) *versus* **BLK_p** conversion (Figure 3-2e) shows a linear relationship and the $\bar{D}$ values of the products remain small (1.06 - 1.12). These results are consistent with the growth of a single copolymer chain through rapid consumption of **DGu_p** followed by slower but contiguous incorporation of **BLK_p**, and thus provide evidence for a ‘block-like’ structure of the resulting copolymer.

A series of poly(Bn-amido-D-glucose)-*block*-poly(Cbz-beta-L-lysine) (**PDGu_p(x)-*b*-PBLK_p(y)**) block copolymers, with varying ratios of x to y but constant target total degree of polymerization of 20, *i.e.* $(x + y) = 20$, was synthesized (Figure 3-2a, Figure 3-3). The molecular weights are close to the design values based on gel permeation chromatography (GPC) relative to polystyrene standards, confirming that the AROP process is well-controlled (Figure 3-2f, Table 3-2).

Figure 3-2. Facile one-shot one-pot synthesis of **PDGu(x)-b-PBLK(y)** block copolymer. **(a)** Synthetic scheme of **PDGu(x)-b-PBLK(y)**. **(b)** *One-shot addition* of both monomers (**DGu_p** and **BLK_p**) leads to block copolymerization when the monomers have contrasting reactivities. **(c to e)** Kinetic studies and **(f)** GPC measurements verify the well-controlled single chain block architecture of **PDGu_p(x)-b-PBLK_p(y)**. **(c)** Remaining monomer concentration vs time. **(d)** GPC

curves of partially polymerized products at selected quenching times. (e) Molecular weight (M_n) and molecular weight distribution (D) as a function of conversion of **BLK_p**. (f) GPC of protected-(co)polymers.

Figure 3-3 ^1H NMR spectra of **PDGu_p(x)-b-PBLK_p(y)** in CDCl_3 .

Table 3-2 Molecular weights and polydispersities of (co)polymers, and ratios of **DGu_p** to **BLK_p** in **PDGu_p(x)-b-PBLK_p(y)**

Sample	Design ratio of DGu _p to BLK _p	Calculated ratio ^a of DGu _p to BLK _p	M _{n,theo} (Da)	M _n , GPC ^a (Da)	PDI ^a
P1	PDGu _p (6.7)-b-PBLK _p (13.3)	PDGu _p (6)-b-PBLK _p (14)	6913	6519	1.10
P2	PDGu _p (8)-b-PBLK _p (12)	PDGu _p (8)-b-PBLK _p (12)	7151	6992	1.09
P3	PDGu _p (10)-b-PBLK _p (10)	PDGu _p (10)-b-PBLK _p (10)	7518	7398	1.11
P4	PDGu _p (12)-b-PBLK _p (8)	PDGu _p (12)-b-PBLK _p (8)	7884	7758	1.12
P5	PDGu _p (13.3)-b-PBLK _p (6.7)	PDGu _p (14)-b-PBLK _p (6)	8122	8074	1.13
P6	PDGu _p (20)	PDGu _p (20)	9350	8278	1.08

After one-step deprotection, the final products **PDGu(x)-b-PBLK(y)** were obtained with overall yields greater than 65% (Figure 3-4). NMR spectroscopy measurements of **PDGu(x)-b-PBLK(y)** show two sets of signals belonging to **PDGu** and **PBLK** respectively, corroborating their block rather than random structures (Figure 3-5 to 3-7).

Figure 3-4 NMR spectra of **PDGu(x)-*b*-PBLK(y)**. **(a)** rt & **(b)** 50 °C ^1H NMR spectra in D_2O .

dy3a_H1
Solvent:D2O

dy3a_C13
Solvent:D2O

dy3a_Dept135
Solvent:D2O

47.16
41.37
39.37
33.14
26.81
22.25

240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

Figure 3-5 ^1H , ^{13}C , DEPT135, COSY and HMQC NMR spectra of **PBLK(20)**.

dy6_C13
Solvent:D2O

— 170.22

74.73
73.10
69.99
68.47
60.37
— 51.09

dy6_Dept135
Solvent:D2O

74.74
73.10
69.99
68.47
60.37
— 51.11

Figure 3-6 ¹H, ¹³C, DEPT135, COSY and HMQC NMR spectra of **PDGu(20)**.

dy35-2_Dept135
Solvent:D2O

74.71
73.12
70.08
68.52
60.29
51.21
47.16
41.40
39.45
33.19
26.99
22.28

240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

Figure 3-7 ^1H , ^{13}C , DEPT135, COSY and HMQC NMR spectra of **PDGu(7)-b-PBLK(13)**.

NMR spectra also show that the ratios of **DGu** to **BLK** in **PDGu(x)-b-PBLK(y)** after purification deviate slightly from the stoichiometric ratios of added monomers. For example, the actual composition of **DGu** and **BLK** in **PDGu(10)-b-PBLK(10)** is **PDGu(7)-b-PBLK(13)**; the **PBLK** block is 66 mole% *versus* the design value of 50 mole%. This trend is repeatable and can be seen in other compositions (Table 3-3). The nomenclature of the polymer followed the actual composition of the final product as calculated from the integration of NMR peaks. For example, the actual composition of **DGu** and **BLK** in the designed **PDGu(10)-b-PBLK(10)** is **PDGu(7)-b-PBLK(13)**, and hence the latter is adopted as the nomenclature of the copolymer.

Table 3-3 Design and actual ratios of **DGu** to **BLK** before and after deprotection.

Sample	Design ratio of DGu _p to BLK _p	Actual ratio ^a of DGu _p to BLK _p	Actual ratio ^b of DGu to BLK after deprotection
P1	PDGu _p (6.7)-b-PBLK _p (13.3)	PDGu _p (6)-b-PBLK _p (14)	PDGu(5)-b-PBLK(15)
P2	PDGu _p (8)-b-PBLK _p (12)	PDGu _p (8)-b-PBLK _p (12)	PDGu(6)-b-PBLK(14)
P3	PDGu _p (10)-b-PBLK _p (10)	PDGu _p (10)-b-PBLK _p (10)	PDGu(7)-b-PBLK(13)
P4	PDGu _p (12)-b-PBLK _p (8)	PDGu _p (12)-b-PBLK _p (8)	PDGu(9)-b-PBLK(11)
P5	PDGu _p (13.3)-b-PBLK _p (6.7)	PDGu _p (14)-b-PBLK _p (6)	PDGu(10)-b-PBLK(10)
P6	PDGu _p (20)	PDGu _p (20)	PDGu(20)

^a Ratios were calculated based on ¹H NMR integrations of (protected) PDGu_p(x)-b-PBLK_p(y)

^b Ratios were calculated based on ¹H NMR integrations of (deprotected) PDGu(x)-b-PBLK(y)

When we attempted the synthesis of **PDGu_p(10)-b-PBLK_p(10)** by the sequential addition of **DGu_p** (M1) followed by **BLK_p** (M2) at −30 °C (Figure 3-8a), the amount of isolated undesired homopolymer **PDGu_p** after the reaction could be more than 50% of the yield (based on **DGu_p**). This sequential copolymerization of higher reactivity **DGu_p** followed by lower reactivity **BLK_p** could be finished rapidly in less than 1 hour, but achieved only low purity block copolymer with substantial **PDGu_p** homopolymer. We expected that by reversing the order of addition of monomers, *i.e.* first **BLK_p** and then **DGu_p** (Figure 3-8b), in which the first block (**PBLK_p**) has a lower reactivity and also a higher transfer rate to **DGu_p**, the block copolymerization would successfully occur. However, the first step requires up to 8 hours to reach ~90% conversion (based on **BLK_p**). In addition, the final mixture was very viscous and contained a large proportion of pre-mature terminated **PBLK_p**. Regardless of the sequence of monomer addition, sequential copolymerization

cannot successfully synthesize cationic glycosylated block copoly(beta-peptide) with good yield and purity. Unexpectedly, with one-shot AROP with simultaneous feed of the two beta-lactams, we could achieve successful synthesis of the block copolymers **PDGu_p-*b*-PBLK_p**.

(a) *Sequential* block copolymerization does not produce block copolymer with high yield and purity when $R^{M2} \ll R^{M1}$ (M1 is blue (DGu_p), M2 is red (BLK_p))

(b) For *sequential* block copolymerization, when M1 polymerization is exceedingly less reactive and results in high viscosity, block copolymers cannot be produced (M1 is red (BLK_p), M2 is blue (DGu_p))

Figure 3-8 *Sequential* block copolymerization approach does not produce block copolymer well for the pair of BLK_p and DGu_p regardless of the sequence of monomer addition. (a) Addition of DGu_p first. (b) Addition of BLK_p first.

Section 3.2 Molecular weight characterization of the polymer series

Besides GPC, mass spectrometry (MS) is often used to characterize the exact molecular weights of polymers. MS techniques such as Electrospray Ionization-Mass Spectrometry (ESI-MS) and Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectroscopy are suitable for polymer characterization due to their nature of soft-ionization and negligible fragmentation²³³. We first attempted to measure the molecular weights using ESI-MS. However, no signal was detected possibly due to the presence of sugar block. It has been reported that polysaccharides potentially suffer from problems of detection because their hydrophilicity make them “sticky” and a lack of charge make them not easily ionizable²³⁴. Since the MS data of poly-amido-saccharides (PAS), which possess similar chemical structure to the sugar block of the copolymer, has been measured by MALDI-TOF²³⁵⁻²³⁶, we adopted the same technique for our polymer series.

The conditions of MALDI-TOF were carefully optimized, including choice of matrix, matrix and sample concentrations (and their ratio), sample spot co-crystallization procedure, machine laser power, number of accumulated scans and other parameters. The molecular weights of the (a) homocationic homopolymer **PBLK(20)**, (b) homosugar **PDGu(20)** and (c) block copolymer **PDGu(7)-b-PBLK(13)** were obtained using α -Cyano-4-hydroxycinnamic acid (CHCA) as the matrix. Signals were obtained in positive ion mode at polymer concentration of 10 mg/mL. The `msbackadj` function in MATLAB[®] Bioinformatics Toolbox[™] was adopted for baseline subtraction.

For homopolymers **PDGu(20)** and **PBLK(20)**, it is obvious that the mass/charge (m/z) distance between each peak is equivalent to the m/z of the respective monomer (Figure 3-9a, b). It is generally understood that the analysis of

block copolymer is more complicated than homopolymer due to the possible variations of each block. Though Gellman *et al* used the m/z of the peak with highest intensity as the representative molecular weight of beta-peptide for simplicity, the author also admitted that this may not be accurate due to possible preferential desorption of smaller polymer relative to larger ones²¹⁸. Hence, in our study we used a more comprehensive method to calculate the number-average molecular weight (Mn) based on the following equation:

$$\bar{M}_n = (\sum m_i N_i) / (\sum N_i)$$

where m_i and N_i are the m/z value and its respective intensity.

The mass spectrometry data were analyzed using a MATLAB[®] based data analysis workflow with functions provided in Bioinformatics Toolbox[™]. The calculated molecular weights Mn are 3012 Da, 3159 Da and 3391 Da for homocationic, homosugar and block copolymer respectively. These values agree well with the design values as well as those measured by GPC (Table 3-4), corroborating the synthesis is well controlled with narrow dispersity.

Table 3-4 Molecular weights Mn (in Daltons) of homocationic, homosugar and block copolymer.

Polymer	Design	MALDI-TOF	GPC	PDI (GPC)
PBLK(20)	2864	3012	3100	1.27
PDGu(20)	3804	3159	3481	1.28
PDGu(7)-<i>b</i>-PBLK(13)	3193	3391	3385	1.41

Figure 3-9 MALDI-TOF data of (a) homocationic **PBLK(20)**, (b) homosugar **PDGu(20)** and (c) block copolymer **PDGu(7)-b-PBLK(13)**.

Section 3.3 Solution properties of the polymer series

The solution properties of the homosugar, homocationic and copolymer were examined by Multi-angle Dynamic Light Scattering in various conditions, including DI water, PBS, 8M urea, and pH = 1, where PBS mimics the physiological salt concentration, urea disrupts the hydrogen-bonding between peptide backbones, and acid promotes complete protonation of side chain amine groups (and hence charge repulsion). Samples were measured at multiple scattering angles (45°, 60°, 75°, 90°, 105°, 120°, 135°, 150°).

Compared with single angle Dynamic Light Scattering, Multi-angle Dynamic Light Scattering measurements are more robust with better reproducibility and accuracy. For a system with stable particle, the following equations are applicable to calculate its hydrodynamic radius (R_h):

To determine R_h from Stokes-Einstein Equation:

$$R_h = \frac{k_B T}{6\pi\eta D_T}$$

where k_B is Boltzmann constant, η is viscosity of the solution, T is absolute temperature in Kelvin (K).

The diffusion coefficient D_T is the gradient of the of decay rate Γ versus wave vector q^2 :

$$D_T = \frac{\Delta\Gamma}{\Delta q^2}$$

The decay rate Γ can be determined from the following equation:

$$\Gamma = \frac{1}{\tau}$$

The wave vector q can be determined from the following equation:

$$q = \frac{4\pi n_0}{\lambda} \sin\left(\frac{\theta}{2}\right)$$

The abovementioned equations can be visualized in a plot of autocorrelation function $\tau A(\tau)$ vs $\log \tau$ for each angle, where the autocorrelation function $\tau A(\tau)$ were obtained using GENDIST package. If a stable particle exists, it should be detected in all angles with all the peaks aligned in a straight line (which corresponds to a well-fitted linear correlation of I vs q^2). If a straight line could not be drawn across all the peaks of different angles, it means the solution does not have stable particle and a reliable R_g (and R_h) could not be determined.

Based on the $\tau A(\tau)$ vs $\log \tau$ plot, the following conditions did not show stable particles: **PBLK(10)**, **PDGu(20)**, **PDGu(7)-*b*-PBLK(13)** in pH1, 8 M Urea, PBS. Hence for these conditions, the values for R_h , R_g , R_h/R_g and PDI were left blank. Although the R_g values can still be calculated from the abovementioned equations, any value falls below 5 nm is considered inaccurate, and hence the solution is considered true solution without stable particle.

The value of R_g/R_h , also known as shape factor, is an indication of the particle morphology, with the typical values shown below:

R_g/R_h	Structure
<0.6	Core-shell
~0.774	Hard sphere
~1	Vesicle
~1.5	Gaussian chain
>2	Long rod

The shape factor and particle morphology of the polymer(s) could change under different conditions.

For homocationic **PBLK(20)**, consistent particle signals were detected in all the angles as shown by the red-dotted line (Figure 3-10a), indicating a stable particle. The polymer existed in the form of particle in all the conditions and formed a core-shell structure, possibly due to hydrophobic interaction between polymer backbone. As the beta-peptides bear one more carbon in the backbone, the hydrophobicity of backbone segment is relatively higher compared to that of alpha peptide. If they were to aggregate via H-bond formation, the aggregation should have been disrupted in 8M Urea. However, stable particle of **PBLK(20)** still existed indicating that the formation of this particle was not due to H-bonding. At pH=1, all the lysine side chains are completely protonated and charge-charge repulsion is maximum, yet stable particle of **PBLK(20)** still exists. Hence the aggregation of the **PBLK(20)** could be due to hydrophobic interaction of the beta-peptide backbone.

However, with the shorter homocationic **PBLK(10)**, particle was only observed in DI water, and the polymer remained as true solution in the presence of PBS, urea and acid (Figure 3-10b). It is speculated that the electrostatic repulsion between the lysine side chains dominated and disrupted the much weaker hydrophobic interactions in the shorter homocationic homopolymer **PBLK(10)**. For hydrophilic homosugar **PDGu(20)** and copolymer **PDGu(7)-*b*-PBLK(13)**, particle with very weak interaction between polymer chains was only observed in DI water, and the particle could be easily disrupted in all other conditions (Figure 3-10 c, d). Though a small particle was observed for copolymer in acidic condition, the particle size was too small to be accurately detected in the DLS measurement, hence it is still considered as true solution. The R_g and R_h were calculated and tabulated in Table 3-5.

Table 3-5 Dynamic Light Scattering measurements of R_g and R_h

Polymer	Condition	R_h (nm)	R_g (nm)	R_g/R_h	PDI
PBLK(20)	DI	79.05	30.9	0.391	0.24
	pH1	74.01	46.4	0.627	0.37
	8 M Urea	92.58	55.1	0.595	0.24
	PBS	47.84	23.3	0.487	0.32
PBLK(10)	DI	45.77	37.5	0.8193	0.33
	pH1	1.25	-	-	-
	8 M Urea	0.95	-	-	-
	PBS	0.87	-	-	-
PDGu(20)	DI	94.7	123.6	1.30	0.16
	pH1	1.01	-	-	-
	8 M Urea	0.64	-	-	-
	PBS	0.9	-	-	-
PDGu(7)-b-PBLK(13)	DI	38.47	30.2	0.785	0.10
	pH1	3.43	-	-	-
	8 M Urea	0.65	-	-	-
	PBS	0.9	-	-	-

Figure 3-10 Dynamic Light Scattering of (a) **PBLK(20)** (b) **PBLK(10)** (c) **PDGu(20)** and (d) **PDGu(7)-b-PBLK(13)** in DI, 8M urea, 1×PBS and pH 1. Normalized relaxation time distribution function $\tau A(\tau)$ (y-axis) vs $\log(\tau)$ (x-axis) at different angles (θ), with each line represents a specific angle of detection θ . From

bottom to top: $\theta = 45^\circ, 60^\circ, 75^\circ, 90^\circ, 105^\circ, 120^\circ, 135^\circ, 150^\circ$. A red dotted line was shown for solution with stable particle.

It is worth noticing that the testing concentration was set at 1 mg/mL for the sake of solution property investigation. Upon further dilution to biologically relevant concentrations (10-100 $\mu\text{g/mL}$ range), all the polymers remained as true solution in the various conditions tested, and no particle was detected as shown in Figure 3-11.

Figure 3-11 Dynamic Light Scattering of (a) **PBLK(20)** (b) **PDGu(20)** and (c) **PDGu(7)-b-PBLK(13)** at 100 $\mu\text{g/mL}$ in PBS. Normalized relaxation time distribution function $\tau A(\tau)$ (y-axis) vs $\log(\tau)$ (x-axis) at different angles (θ), with each line represents a specific angle of detection θ . No stable particle could be detected in all the polymer solutions.

Chapter 4 Antibacterial properties and mechanism of action study of the glycosylated cationic block co(beta-peptides)

Antimicrobial resistance (AMR) has become a chronic and severe problem for global health. New mechanisms of resistance against last-resort antibiotics continue to emerge and spread globally²³⁷. It is estimated that by 2050, AMR will surpass cancer and become the biggest killer, resulting in 10 million deaths per year, and that the cumulative global economic cost by then will reach 100 trillion USD⁹. This gloomy prospect is exacerbated by the decline in investment and reduced interest in R&D for antibiotic discovery on the part of both pharmaceutical industry and public funders²³⁸. The rapid development and spread of resistance globally, coupled with the “discovery void” of new type of antibiotics in the past three decades, urgently calls for alternatives to antibiotics to treat “superbugs”. The World Health Organization (WHO) recently published a priority list of bacteria for which new antibiotics are urgently needed⁸. Methicillin-resistant *Staphylococcus aureus* (MRSA), a WHO high priority pathogen, is a leading cause of mortality due to antibiotic-resistant infections²⁴⁻²⁵. Initially restricted to hospitals and healthcare settings, MRSA is causing an increasing number of infections in the community²³⁹⁻²⁴⁰. MRSA is associated with poor clinical outcomes²⁴¹; causing frequent skin and soft tissue infections²⁴² and can disseminate, resulting in life-threatening bloodstream infections, endocarditis, bone and joint infections, as well as pneumoniae²⁴³⁻²⁴⁴.

The resulting optimized block co-beta-peptide, **PDGu(7)-block-PBLK(13)**, is non-cytotoxic and non-hemolytic *in vitro*. Further, the block co-beta-peptide has interesting biological properties, which are further elaborated in detail in this and the subsequent chapters.

Section 4.1 *In vitro* biocompatibility

Since toxicity towards mammalian cells is a major drawback for antimicrobial peptides/polymers, we first examined the biocompatibility of the **PDGu(x)-b-PBLK(y)** polymer series. The *in vitro* cytotoxicity of (co)polymer series against 3T3 fibroblast cells was tested and plotted as cell viability % vs concentration in Figure 4-1. The homocationic **PBLK(20)** was very toxic, and the toxicity gradually decreased with the increase of **PDGu** ratio. The values of IC₅₀ (concentration causing 50% death of fibroblast cells) were listed in Table 4-1.

Figure 4-1 *In vitro* cytotoxicity assay. **PDGu(x)-b-PBLK(y)** series 24-hour acute toxicity against 3T3 mouse fibroblast cells.

Besides cytotoxicity to fibroblast cells, the hemolytic activity towards erythrocytes are commonly tested for antimicrobial polymers. The definition of hemolytic activity is ambiguous, with variations in the literature for quantification, such as HC₁₀₀, HC₅₀, HC₁₀ values representing the concentration that causes 100%, 50% or 10% lysis of red blood cells respectively.

In our study, we chose HC_{10} , which represents the minimal hemolytic concentration, to investigate the hemolytic activity of the polymer series. The hemolytic activity was tested and plotted as Hemolysis % vs concentration in Figure 4-1 and HC_{10} values were shown in Table 4-1. The hemolytic activity is minimal up to 20,000 $\mu\text{g/mL}$ ($< 10\%$ hemolytic) for **PDGu(x)-b-PBLK(y)** polymer series when $y \leq 13$. Higher **PBLK** content reduces hemocompatibility, as shown by the dramatic increase of hemolytic activity when concentration goes beyond 2,500 $\mu\text{g/mL}$.

To evaluate whether the difference in hemolytic profile were due to charge density, the zeta potential of the homocationic, copolymer and homosugar were recorded in PBS to mimic physiological conditions. The zeta potential value of **PBLK(20)** were slightly higher than that of **PDGu(7)-b-PBLK(13)** in PBS (16.6 mV vs 12.6 Mv, also shown below). However the difference was subtle, indicating that the difference of hemolytic activity were not directly linked to charge density. Furthermore, the homosugar **PDGu(20)** exhibited slightly negative zeta potential, yet its hemolytic profile was comparable to that of the co-beta-peptide **PDGu(7)-b-PBLK(13)** with positive zeta potential. This corroborated that zeta potential is less relevant to hemolytic profile in this polymer series.

Rather, we believed that the hemolytic activity was closely correlated to the hydrophobicity of the compounds. Many antimicrobial polymers and peptidomimetics are intrinsically amphiphilic with exposed hydrophobic domains in free solution and are typically hemolytic because their freely exposed hydrophobic moieties would interact with erythrocytes¹⁵⁷. However, our copolymer is hydrophilic without distinct hydrophobic domain. The amine group of the cationic block dominates the interactive topology with erythrocytes but its hydrophilicity minimizes hemolysis. Further, the neutral sugar block also increases the hydrophilicity of the block copolymer. The excellent hemocompatibility makes this copolymer series attractive for further development.

Earlier versions of antimicrobial beta-peptides by Gellman *et al* and Degrado *et al* are made of hydrophobic monomers and are hemolytic, with HC₅₀ values in the range of 10 to 100 µg/mL^{197, 202-203}. In their subsequent studies, incorporation of neutral or hydrophilic beta-lactam components in the beta-peptide system further reduced the hemolytic activity (HC₁₀) to a range of 100 to 1000 µg/mL²⁴⁵. When a relatively more hydrophilic monomer BLK was adopted by our study, even the homocationic **PBLK(20)** showed better hemolytic activity, with HC₁₀ value of 5000 µg/mL.

Figure 4-2 Hemocompatibility of **PDGu(x)-b-PBLK(y)**

Table 4-1 Hemolytic activity and mammalian cell biocompatibility of the (co)polymer series.

Sample	HC ₁₀ (µg/mL)	IC ₅₀ (µg/mL)
	<i>Red blood cell</i>	<i>fibroblast 3T3 cell</i>
PBLK(20)	5,000	18
PDGu(5)-b-PBLK(15)	3,300	100
PDGu(6)-b-PBLK(14)	4,800	150
PDGu(7)-b-PBLK(13)	>20,000	430
PDGu(9)-b-PBLK(11)	>20,000	395
PDGu(10)-b-PBLK(10)	>20,000	630
PDGu(20)	>20,000	>1,024

Section 4.2 Minimal Inhibitory concentration (MIC) of (co)polymer series

The **PDGu(x)-b-PBLK(y)** series was tested against a panel of Gram-positive bacteria (Table 4-2). Though the homocationic **PBLK(20)** showed the best antimicrobial activity, it was not selective, being cytotoxic to eukaryotic cells and also hemolytic as discussed in Section 4.1. The copolymer **PDGu(7)-b-PBLK(13)**

showed the most balanced profile, combining potency against *S. aureus* with good selectivity index (>25) and no hemolysis (HC₁₀ > 20,000 µg/mL). Hence **PDGu(7)-b-PBLK(13)** was chosen as the focus and was referred to as the “copolymer” for subsequent studies.

Table 4-2 Antimicrobial activity (measured as MIC₉₀) of the (co)polymer series.

Sample	MIC ₉₀ (µg/mL)					HC ₁₀ (µg/mL) <i>RBC</i>	IC ₅₀ (µg/mL) <i>3T3</i>
	SA 25923	SA 29213	MRSA BAA40	MRSA USA300	B. <i>subtilis</i>		
PBLK(20)	8	8	8	8	4	5,000	18
PDGu(5)-b-PBLK(15)	8	8	8	8	4	3,300	100
PDGu(6)-b-PBLK(14)	16	8	8	8	4	4,800	150
PDGu(7)-b-PBLK(13)	16	8	8	8	4	>20,000	430
PDGu(9)-b-PBLK(11)	32	16	16	16	8	>20,000	395
PDGu(10)-b-PBLK(10)	64	32	32	32/64	16	>20,000	630
PDGu(20)	>512	>512	>512	>512	>512	>20,000	>1,024

Besides the two methicillin-sensitive *S. aureus* strains, the copolymer shows good activity against MRSA USA300 (Table 4-2), the predominant community-associated (CA-)MRSA²³⁹. Inspired by the potency against USA300, we also tested the copolymer against several (17) well-characterized hospital-associated (HA-)MRSA strains representing the major lineages (Table 4-3). This is clinically significant because multi-drug resistant (MDR) HA-MRSA bacteria cause the majority of nosocomial bacteraemia/ septicaemia and device-related infections involving biofilm formation. Our copolymer shows similar activity against MRSA USA300 as against the (17) (HA-)MRSA strains which are resistant to multiple conventional antibiotics including vancomycin and daptomycin. The copolymer MICs against all the 17 new HA-MRSA strains tested (8-16 µg/mL) are comparable to that for MRSA USA300 (16 µg/mL); the 17 MRSA strains included 7

vancomycin-resistant *S. aureus* (Strains #1 to 7), 7 daptomycin non-susceptible vancomycin-intermedia²⁴⁶ *S. aureus* (Strains #8 to 14), and 3 multi-drug resistant (MDR) MRSA strains (Strains #15 to 17). (The detailed characterization of the strains was listed in Appendix 4). The results showed that our copolymer is just as effective against HA-MRSA strains with resistance to multiple conventional antibiotics (Table 4-3).

A particularly interesting phenomenon was the activity of copolymer against daptomycin-resistant MRSA strains (Table 4-3). Daptomycin has been used to treat persistent bacteraemia as an alternative to vancomycin, especially when vancomycin insensitive strains are involved²⁴⁷. Daptomycin resistance involves changes in membrane lipid composition (gain of function *MprF* mutants) which result in an increase in lysyl-phosphatidyl glycerol and hence an increased positively charged membrane⁴⁹⁻⁵⁰. Hence daptomycin resistant strains often carry cross-resistance to other cationic antimicrobial peptides⁴⁷. Indeed, *MprF* mutation were identified in Strains #10 to 13 of Table 4-3. The copolymer retained its MIC against these DAP-resistant clinical strains, further confirming its activity against *MprF* mutants. This also indicated the mechanism of action is not completely the same as daptomycin and many cationic antimicrobial peptides (to be discussed in later sections).

We further tested the copolymer **PDGu(7)-b-PBLK(13)** against 15 ESKAPE pathogens and other clinically relevant Gram-positive bacteria. The copolymer was active against the Gram-positive *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus epidermidis* at concentrations comparable to that for *S. aureus* (Strains #1-4, Table 4-4). However, the copolymer is 4 to 16 times less potent against *Enterococci* and *Streptococci* (Strains #5-11, Table 4-4), and is 8

to 32 times less potent against the Gram-negative bacteria tested (Strains #12-15, Table 4-4).

Table 4-3 Antimicrobial activity of **PDGu(7)-b-PBLK(13)** against multi-drug resistant clinically isolated MRSA.

	Serial No.	Designation	MIC (µg/mL)		Multi-drug resistance	Major lineage/ Clonal Complex ⁴⁸
			PDGu(7)-b-PBLK(13)	Resistant Antibiotic		
VAN resistant <i>S. aureus</i>	1	HIP11714	16	512	CIP, CLI, ERY, GEN, LVX, MXF, OXA, RIF, TEC	5
	2	HIP11983	16	16	CIP, CLI, ERY, GEN, LVX, MXF, OXA, TET	5
	3	HIP13170	16	128	CIP, CLI, ERY, GEN, LVX, MXF, OXA, TEC, TET	5
	4	HIP13419	16	VAN 64	CIP, CLI, ERY, GEN, LVX, MXF, OXA, TEC, TET	5
	5	HIP14300	16	32	CIP, CLI, ERY, LVX, MXF, OXA, TEC	5
	6	HIP15178	16	512	CIP, CLI, ERY, LVX, MXF, OXA, TEC	5
	7	AIS2006032	16	>512	CIP, CLI, ERY, LVX, MXF, OXA, TEC	5
DAP non-susceptible VAN intermediate <i>S. aureus</i>	8	HIP09433	16	4	CIP, ERY, GEN, LVX, MXF, OXA, PEN, TMP	45
	9	SAMER-S6	16	16	TMP, PEN, TEC	5
	10	6820	16	8	OXA, RIF, TEI	5
	11	TTSH-478700	8	DAP 16	CIP, LVX	22
	12	TTSH-671549	16	8	CIP, ERY, LVX	22
	13	TTSH-478701	8	4	CIP, ERY, LVX, RIF	22
	14	ATCC 700789	16	4	CIP, ERY, LVX, RIF, TOB	5
MDR MRSA	15	ATCC BAA38	16	128	PEN, STR	8
	16	ATCC BAA39	16	TET 128	CIP, ERY, GEN, IPM, LVX, PEN, TMP, TOB	8
	17	ATCC BAA44	16	32	CIP, ERY, GEN, LVX, PEN, TOB	8

CIP, ciprofloxacin; CLI, clindamycin; DAP, daptomycin; ERY, erythromycin; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; MXF, moxifloxacin; OXA, oxacillin; PEN, penicillin; RIF, rifampicin; STR, streptomycin; TEC, teicoplanin; TET, tetracycline; TMP, trimethoprim; TOB, tobramycin; VAN, vancomycin

Table 4-4 MIC values of **PDGu(7)-b-PBLK(13)** against ESKAPE pathogens and other clinically relevant Gram-positive bacteria.

Strain No.	Bacteria strains (Gram-positive)	Designation	Copolymer MIC (µg/mL)
1	<i>Bacillus cereus</i>	ATCC 11778	16
2	<i>Listeria monocytogenes</i>	ATCC 19115	16
3	<i>Staphylococcus epidermidis</i>	ATCC 35984	8
4	<i>Staphylococcus epidermidis</i>	ATCC 700563	8
5	<i>Enterococcus faecium</i>	ATCC 29212	128
6	<i>Enterococcus faecalis</i>	VRE V583	256
7	<i>Streptococcus agalactiae</i>	ATCC 13813	64
8	<i>Streptococcus iniae</i>	ATCC 29178	128
9	<i>Streptococcus parasanguinis</i>	ATCC 15912	64
10	<i>Streptococcus pneumoniae</i>	ATCC BAA334	256
11	<i>Streptococcus pyogenes</i>	ATCC 12344	64
Bacteria strains (Gram-negative)			
12	<i>Acinetobacter baumannii</i>	ATCC 19606	256
13	<i>Escherichia coli</i>	ATCC 25922	128
14	<i>Klebsiella pneumoniae</i>	ATCC 12993	512
15	<i>Pseudomonas aeruginosa</i>	ATCC 27853	512

Section 4.3 Kill kinetics of the copolymer **PDGu(7)-b-PBLK(13)**

Since we have shown the copolymer selectively killed MRSA, we conducted time kill assay to study the kill kinetics of copolymer against MRSA USA300 (Figure 4-3). Vancomycin, the last-resort antibiotics for MRSA infection, was used as control. With 4×MIC and 2×MIC treatment of **PDGu(7)-b-PBLK(13)**, 100% killing was achieved in 1 hour and 2 hours respectively. For comparison, 100% killing with vancomycin required 7 hours at 4×MIC and 24 hours 2×MIC. In general, **PDGu(7)-b-PBLK(13)** showed both time and concentration dependent killing

kinetics, while vancomycin showed much slower killing, as has been previously reported.²⁴⁸⁻²⁴⁹

Compared with bacteriostatic antibiotics, bactericidal antibiotics are preferred in many situations such as endocarditis, meningitis, osteomyelitis and neutropenia, where clinical studies have proven that improved outcome and therapeutic efficacy was achieved with bactericidal agents²⁵⁰. Moreover, bactericidal agents could minimize the emergence of resistance in clinical usage, due to minimized bacterial gene upregulation, mutation or both²⁵¹. The fast-killing bactericidal activity could be an advantage of **PDGu(7)-b-PBLK(13)** due to its true sterilization effect within a short period of time at a relatively low concentration (2×MIC).

Figure 4-3 Time-dependent killing of actively growing MRSA USA300 at 4×MIC, 2×MIC, 1×MIC and 0.5×MIC of (purple, a) **PDGu(7)-b-PBLK(13)** and (black, b) vancomycin in MHB.

Section 4.4 Bacterial resistance development towards copolymer

The rapid evolution of resistance towards last resort of antibiotics is particularly worrisome, and an ideal antibacterial should have a low propensity to

elicit resistance development²³⁷. We used two separate assays, *i.e.* spontaneous mutants selection and resistance development by serial passaging, to prove the resistance development against the copolymer is extremely difficult.

Spontaneous mutants selection

A large inoculum of MRSA USA300 cells ($>10^9$ CFU) were exposed to $10\times$ MIC of copolymer for a prolonged period. Polymer solution was changed every 48 hours during the incubation and the OD_{600nm} values were recorded daily over 6 days. At the end of the test, polymer-treated sample remained clear and no escape mutants could be selected (Figure 4-4). Representative images of the copolymer treated sample tubes were shown in the lower panel of Figure 4-4. The culture from day 6 was plated onto agar plates, and no colony was spotted after 48-hour incubation. The selection of escape mutants to **PDGu(7)-b-PBLK(13)** at $10\times$ its MIC was unsuccessful, indicating that the propensity for emergence of resistance is extremely low (frequency below 3×10^{-10} , which is much lower than reported values for antibiotics²⁵²⁻²⁵³).

Figure 4-4 Selection of spontaneous escape mutants to copolymer at $10\times$ MIC

Resistance development by serial passaging

We also attempted to evolve resistance in MRSA USA300 by daily serial passaging in the presence of sub-inhibitory concentrations of copolymer or antibiotic control (as described previously²⁵⁴). This approach also did not select for copolymer-resistant colonies of MRSA USA300. During the 14-day test, no resistance to the copolymer was observed (with only a 2-fold increase in MIC) (Figure 4-5). Resistance was not observed in any of the colonies tested on the 14th day. Conversely, resistance to the antibiotic controls (ciprofloxacin and daptomycin) was rapidly selected for. The test was done with three independent biological replicates.

Figure 4-5 Resistance development of MRSA USA300 by serial passage with sub-inhibitory concentrations of the agent. Data are presented as folds MIC change over time. (a) PDGu(7)-b-PBLK(13), (b) Ciprofloxacin antibiotic control and (c) Daptomycin antibiotic control. All tests were done with three biological replicates.

To further elucidate the low propensity of resistance development against copolymer, we also tested the resistance evolution of two more HA-MRSA strains (MRSA BAA38 and BAA40). Similarly, resistance against ciprofloxacin control (Figure 4-6c and d) and daptomycin control (Figure 4-6e and f) rapidly evolved, whilst copolymer-resistant MRSA colonies could not be selected (Figure 4-6, a and b).

Figure 4-6 Resistance development of (a, c, e) MRSA BAA38 and (b, d, f) BAA40 by serial passaging with sub-inhibitory concentrations of the agent. Data are presented as folds MIC change over time. (a,b) PDGu(7)-b-PBLK(13), (c,d)

Ciprofloxacin antibiotic control and **(e,f)** Daptomycin antibiotic control. All tests were done with three biological replicates.

In summary, the copolymer mutation frequency is much lower than that of the classical antibiotic ciprofloxacin even though we cannot be completely certain that selection of escape mutants resistant to the copolymer is not possible. It is possible that emergence of resistance requires several independent mutations, which could not be observed under our experimental design. Nevertheless, we could conclude that the propensity of resistance development against the copolymer was much lower than antibiotics.

Although several reviews have reported the resistance development towards antimicrobial peptides (AMPs) and the associated mechanism of resistance (MoR), it is generally true that polymeric or peptide-based antimicrobials targeting bacterial membranes are less prone for bacterial resistance development²⁴⁶. Specifically, peptidomimetics have been shown to trigger no resistance development in a similar experimental setup²⁵⁵⁻²⁵⁷, corroborating with our finding. The low propensity of resistance development towards our co-beta-peptide is attributed to its fast kill-kinetics and its multiple targets present in the bacteria (cell envelope including cell membrane and cell wall teichoic acid), which is elaborated in details in the subsequent sections.

Section 4.5 Mechanism of Action (MoA) study

The structure of the cationic block co-beta-peptide suggests a possible mechanism of action involving membrane interaction. Confocal microscopy of fluorescently-labelled bacterial cells showed that the rhodamine-labelled **PDGu(7)-**

***b*-PBLK(13)** accumulated preferentially in the bacteria envelope (*i.e.* cell wall and cell membrane) (Figure 4-7a). The rhodamine-labelled **PBLK(20)** accumulated both in the bacterial membrane and inside bacterial cells (Figure 4-7b). This indicated that incorporation of sugar block potentially affected the MoA of the polymer.

Figure 4-7 Confocal image of (a) copolymer **PDGu(7)-*b*-PBLK(13)** and (b) homocationic **PBLK(20)** treated 10^8 CFU/mL MRSA at $1\times$ MIC for 1 hour. Scale bar = 1 μ m. From left to right: (I) Rhodamine-labelled polymer channel, (II) FM1-43 labelled bacteria membrane channel, (III) superimposed images from both channels.

The effect of **PDGu(7)-*b*-PBLK(13)** on the morphology of MRSA USA300 was also visualized by cryo-transmission electron microscopy (cryo-TEM), which revealed a much larger periplasmic space gap (of about 7-8nm, Figure 4-8a;

indicated by red arrows), together with bleb and vacuole formation (Figure 4-8a). In contrast, periplasmic gap widening, blebs, and vacuoles were not observed in untreated bacteria (Figure 4-8b). Treatment with **PBLK(20)** led to significant bacterial envelope deformation, cell leakage and lysis (Figure 4-8c).

The accumulation of the block co-beta-peptide **PDGu(7)-b-PBLK(13)** at the outer leaflet of the cytoplasmic membrane causes the increased periplasmic space visible in the cryo-TEM (Figure 4-8a), which led to detachment of the cell wall from the cytoplasmic membrane, causing a weakened membrane-cell wall interface. The copolymer also aggregates inside the cell wall leading to defects in the cell wall function. The blebs observed with copolymer treatment using cryo-TEM may be formed by membrane-bound cytoplasm herniating through cell wall defects as intracellular water expands during the freezing process of the cryo-TEM preparation²⁵⁸⁻²⁵⁹ (Figure 4-8a II, III). The vacuoles observed (Figure 4-8a) may be ice pockets formed during the cryo-TEM process as water migrates to the polymer-rich periplasmic space since the cytoplasmic membrane is detached from the cell wall.

Taken together, the copolymer disturbs the cell envelope which includes the membrane, the membrane-cell wall interface, and also the cell wall without causing significant pore formation/deformation of the cells. On the other hand, the homocationic **PBLK(20)**, significantly disrupted the cell envelope, leading to leakage/rupture of the bacteria cells. In the subsequent contents of this section, the effect on cytoplasmic membrane and cell wall were discussed in detail.

Figure 4-8 Cryo-TEM images of (a) **PDGu(7)-b-PBLK(13)**, (b) untreated control and (c) **PBLK(20)** treated 10^8 CFU/mL MRSA USA300 in PBS. I, II and III are three representative sets of images. The right panels are zoom-in images of the left panels.

4.5.1 Block co-beta-peptide targets bacterial membrane

Membrane damage was confirmed using propidium iodide (PI) as a marker of plasma membrane integrity (Figure 4-9a). PI dye (red) is excluded from intact bacterial cells and can only stain cells with damaged membrane. Results showed that both the block copolymer and cationic homopolymer are membrane active, but the copolymer induces less PI staining, suggesting that it is less membrane-lytic (Figure 4-9a). DiSC₃₅ dye assay, which probes plasma membrane potential changes, corroborated the finding that **PDGu(7)-b-PBLK(13)** mildly depolarized the bacterial plasma membrane, unlike the homocationic **PBLK(20)** that had a more pronounced effect (Figure 4-9b). Together, the PI staining and DiSC₃₅ assay results indicate that the copolymer disturbs the bacterial membrane without causing severe leakage. These data corroborated well with the microscopic studies indicated previously.

Figure 4-9 (a) Flow cytometry study of propidium iodide stained MRSA USA300. (I) Live bacteria control, (II) bacteria treated with 1×MIC **PDGu(7)-b-PBLK(13)**, (III) 4×MIC **PDGu(7)-b-PBLK(13)**, (IV) 1×MIC **PBLK(20)**, and (V) 4×MIC **PBLK(20)**. (b) DiSC₃₅ membrane depolarization assay. Data are presented as mean ± standard deviation of three measurements.

Selectivity towards anionic membrane liposome over zwitterionic liposome

Conformation change upon interaction with bacterial membrane is a commonly observed phenomenon with membrane-active AMPs²⁶⁰. We further conducted circular dichroism (CD) measurements of the homosugar **PDGu(20)**, homocationic **PBLK(20)** and copolymer **PDGu(7)-b-PBLK(13)**, to elaborate their secondary structure change in the presence of liposome model membrane systems. Similar technique has been used to probe the binding interaction between antimicrobial beta-peptide and bacterial membrane mimicking liposomes²⁰³⁻²⁰⁴.

The molar ellipticities of each polymer were obtained under various conditions, *i.e.* DI water, 10 mM phosphate buffer (pH 2.6-8.7), 20 mM carbonate buffer (pH 10.8) and in the presence of POPG or POPC liposomes. The homosugar **PDGu(20)** adopted a left-handed helical structure in all conditions due to the conformational constraints from the ring structure of the DGu monomer²⁶¹ (Figure 4-10a). In free solution, electrostatic repulsion between the lysine side chains of the cationic **PBLK(20)** causes it to adopt a random coil conformation (Figure 4-10b). However, in the presence of anionic liposomes of POPG which mimics bacterial membrane, the positive charges in the **PBLK** side chains are neutralized by anionic bacterial lipids so that lysine side chain charge-charge repulsion causing the distortion of the helical conformation is substantially reduced. As a result, **PBLK(20)** transforms to a left-handed helix conformation as indicated by a pronounced minimum at 213 nm, a zero crossing at 203 nm and a maximum at 196 nm (Figure 4-10b)²⁶²⁻²⁶³. It is known that beta peptides containing cyclic beta-amino acids adopt different helical structures to those containing non-cyclic amino acids²¹². From the CD spectroscopy data, we see that (cyclic) PDGu spectral exhibited minima at

220nm while the (non-cyclic) PBLK spectral exhibited minima at 213nm (Figure 4-10 a and b). At pH 10.8, **PBLK(20)** became marginally soluble due to deprotonation of the lysine side chains, and a more pronounced helical structure was formed. It differed from the helical structure induced by anionic POPG liposomes, possibly due to the aggregation of the polymer chains. In the presence of zwitterionic POPC liposome which mimics mammalian cells, **PBLK(20)** remained as random coil, indicating a lack of interaction with POPC liposome (Figure 4-10b).

In free solution, circular dichroism (CD) spectroscopy of the block co-beta-peptide adopts a helix-coil conformation attributed respectively to the sugar²⁶¹ and cationic²⁶⁴ blocks (Figure 4-10c). In the presence of anionic liposomes, **PDGu(7)-b-PBLK(13)** copolymer also shows a more pronounced left-handed helical structure compared to that in water and it is likely that the cationic block transitions to the left-handed helical structure (Figure 4-10c). Similarly, no secondary structure transition was observed in the presence of zwitterionic POPC liposomes. The lack of interaction with mammalian cell mimicking liposome corroborated with the excellent biocompatibility of the copolymer as discussed in Section 4.1.

Figure 4-10 Molar ellipticity $[\theta]$ circular dichroism spectra of (a) **PDGu(20)**, (b) **PBLK(20)** and (c) **PDGu(7)-b-PBLK(13)** at 0.05 mg/mL in different media i.e. DI water, 10 mM phosphate buffer (pH 2.6-8.7), 20 mM carbonate buffer (pH 10.8) and in the presence of POPG or POPC liposomes.

To investigate the partitioning of beta-peptides between membrane and aqueous phases, we further conducted the CD measurements at low (5:1) to high (around 1:80) Peptide:Lipid (P:L) ratios while keeping the peptide concentration constant (0.05 mg/mL) (Figure 4-11). The CD signal increases as the P:L ratio increases but it plateaus at the charge neutralization ratio and beyond; the charge neutralization P:L ratios for the homocationic **PBLK(20)** and the copolymer **PDGu(7)-b-PBLK(13)** are 1:20 and 1:13 respectively. At the P:L ratio used in Figure 4-10, complete partition of peptide to anionic membrane lipids was observed, hence the chosen ratio for Figure 4-10 was appropriate.

Figure 4-11 Molar ellipticity $[\theta]$ circular dichroism spectra of (a) **PDGu(20)**, (b) **PBLK(20)** and (c) **PDGu(7)-b-PBLK(13)** in the presence of anionic POPG liposome at different P:L ratios.

To investigate the driving force of the copolymer binding to bacterial membrane, we further conducted Isothermal Titration Calorimetry (ITC) measurements (Figure 4-12). The copolymer showed a thermodynamically favorable binding to anionic POPG liposomes, as indicated by the negative ΔG (Table 4-5, Figure 4-12a, b). The binding is favored by strong electrostatic interaction and opposed by entropy. Upon binding to liposomes, the copolymer undergoes transition from helix-coil to helix-helix, resulting in a more ordered packing of the molecule and hence a decreased (unfavorable) entropy²⁶⁵. The strong electrostatic interaction between the positively charged copolymer and negatively charged membrane lipid outweighs the entropy loss, resulting in strong binding.

(Table 4-5). No interaction with zwitterionic POPC liposomes were observed (Figure 4-12 c, d), corroborating with the CD data.

Table 4-5 Summary of thermodynamic parameters of copolymer with anionic POPG and zwitterionic POPC as determined by ITC

	ΔG (kcal/mol)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	K_d (M) e^{-6}
POPG	-7.13	-12.6	5.43	9.48 ± 3.92
POPC	-	-	-	-

Figure 4-12 Summary of (a, c) heat flow ($\mu cal/s$) change with time and (b, d) associated heat of reaction as determined by isothermal titration calorimetry. 0.15mM copolymer (in syringe) titrated to (a, b) 5mM POPG liposomes or (c, d) 5mM POPC liposomes. The heat of reaction was determined by integration of the calorimeter traces and subtracting the peptide-into-buffer control experiment.

In summary, the CD and ITC data confirmed the interaction of copolymer with anionic bacterial membrane. However, unlike homocationic **PBLK(20)**, the copolymer did not incur significant membrane depolarization or cytoplasmic membrane disruption and accumulated preferentially in the cell envelop which includes the cell wall, membrane and wall-membrane interface. The interaction of copolymer with cell wall components are discussed in the next section.

4.5.2 Block co-beta-peptide targets wall teichoic acid

Wall teichoic acid (WTA) is a group of negatively charged glycopolymers abundantly present in the cell surface of Gram-positive bacteria²⁶⁶. It is the most abundant polyanion in bacterial cell envelope, accounting as much as 60% of cell wall dry mass and forms a dense network of negative charges on Gram-positive cell surfaces²⁶⁷⁻²⁶⁸. WTA is covalently linked to peptidoglycan and is important for cell function, modulating cell surface charge, elasticity, porosity, tensile strength and membrane permeability²⁶⁷. Together with a thick layer of peptidoglycan, WTA serves as a scaffold for extracytoplasmic enzymes (such as cell wall hydrolyase) to control cell division and elongation²⁶⁹⁻²⁷¹. It is also important for metal ion homeostasis²⁷², biofilm formation²⁷³⁻²⁷⁴, host tissue adhesion and pathogenicity²⁷⁵⁻²⁷⁷. Recently, it has been shown to be responsible for beta-lactam antibiotic resistance in MRSA²⁷⁸⁻²⁷⁹. WTA is a relatively underexplored target to tackle antimicrobial resistance. In the past decade, small molecules targeting the synthesis pathways of WTA have been developed and their antimicrobial efficacy has been proved both *in vitro* and *in vivo*²⁸⁰⁻²⁸⁴. However, problems of mammalian cell toxicity and resistance development towards these small molecules remain to be solved.

In this section, we further investigated its mechanism of action (MoA) using WTA-deficient mutants (LAC* Δ tagO) and showed that WTA is an indispensable target. The susceptibility of LAC* Δ tagO is significantly (16-fold) reduced compared with wild type LAC*. Transmission Electron Microscopy (TEM) of sectioned MRSA cells revealed evidence of different MoAs in wild type and WTA-deficient mutants. The co-beta-peptide also potentiates beta-lactam antibiotic oxacillin against 4 strains of hospital-associated (HA-)MRSA, corroborating its WTA-targeting MoA.

Susceptibility of teichoic acid deficient MRSA mutants

As mentioned above, WTA contributes significantly to the anionic charges in Gram-positive bacteria²⁶⁶. Hence we hypothesized that the cationic co-beta-peptide could target WTA by electrostatic interaction, similar to some reported cationic polymers such as branched poly(ethyleneimine) (BPEI)²⁸⁵ and chitosan²⁸⁶. To prove this, we conducted MIC tests of the co-beta-peptide and various types of antibiotics against WTA- and Lipoteichoic acid (LTA)-deficient MRSA mutants (Table 4-6). TagO is the first gene involved in WTA synthetic pathway that initiates the assembly of WTAs, and tagO deletion completely abrogates WTA production. Compared to wild type LAC*, the co-beta-peptide showed significantly (16-fold) increased MIC against the WTA-deficient strain LAC* Δ tagO. The MICs *versus* LTA-deficient strains (LAC* Δ ltaS ANG2414 and LAC* Δ ltaS ANG2434) were the same as for the wild type. This indicates that the killing mechanism of the co-beta-peptide *versus* the wild type depends on WTA, rather than LTA.

Interestingly, the teichoic acid deficient mutants were re-sensitized to beta-lactam antibiotics (oxacillin, penicillin) (with 64- and 8-fold decrease in MIC

respectively), corroborating the function of teichoic acid in beta-lactam antibiotic resistance. Antibiotics from other categories showed unchanged MIC against the mutants, indicating that teichoic acids did not interact with those antibiotics. It also showed that the genetic modification to generate teichoic acid mutants did not lead to secondary effects to alter other functionalities such as bacterial cell membrane and intracellular activities targeted by the tested antibiotics.

Table 4-6 Susceptibility of copolymer and antibiotics in teichoic acid deficient MRSA mutants

Compound	Target	Wild type	WTA-deficient	LTA-deficient	
		LAC*	LAC* Δ tagO	LAC* Δ taS ANG2414	LAC* Δ taS ANG2434
PDGu(7)-b-PBLK(13)	Cell envelope	16	256	16	8
Gentamicin	Intracellular targets	0.5	0.5	0.5	0.5
Ofloxacin		8	8	8	8
Nisin	Bacterial membrane	128	128	128	64
Daptomycin		2	2	2	1
Oxacillin	Cell wall synthesis (Penicillin binding protein)	8	0.125	0.125	0.125
Penicillin		1	0.125	0.125	0.125

TEM imaging of wild type and WTA-deficient MRSA with co-beta-peptide treatment

To visualize the effect of co-beta-peptide treatment on cell morphology, sectioned MRSA cells were imaged with TEM. A ruthenocene dye which did not interfere with cellular activity was covalently attached to the co-beta-peptide using

published protocol²⁸⁷. The ruthenocene-labelled co-beta-peptide is electron-dense in TEM imaging (Figure 4-13). Compared to the untreated control LAC* cells showing a thin, well-aligned cell wall (Figure 4-13a), the co-beta-peptide accumulated preferentially at WTA-rich outer peptidoglycan cell wall layer, resulting in a significantly thickened outer layer of cell wall (Figure 4-13b, white arrows) and rougher surface in the treated LAC* cells (Figure 4-13b). The co-beta-peptide also accumulated at the outer leaflet of the cell membrane (Figure 4-13b, red arrows). The accumulation of co-beta-peptide in cell wall and membrane is attributable to the electrostatic interaction between cationic peptide and anionic components in cell envelope (WTA and cytoplasmic membrane). The lack of pore formation is attributed to the double hydrophilic nature of the cationic and glycosylated blocks of co-beta-peptide, which does not penetrate deep into the hydrophobic core of the membrane bilayer. The roughened surface could be a result of co-beta-peptide interaction with WTA which leads to defects in the integrity of WTA²⁷¹ (Figure 4-13e).

We also imaged co-beta-peptide treated LAC* Δ tagO with TEM. As previously reported²⁷¹, the WTA-deficient LAC* Δ tagO showed irregular shape and misplaced asymmetrical septa, indicating cell separation defects due to absence of WTA (Figure 4-13c). At 1 $\times$ MIC co-beta-peptide treatment, significant cell lysis and membrane deformation were observed (Figure 4-13d), indicating that the killing mechanism of the co-beta-peptide in LAC* Δ tagO is different from that in wild type LAC*. These results, together with the susceptibility test, confirmed our hypothesis that WTA is the primary target of the co-beta-peptide in wild-type MSRA.

Figure 4-13 TEM image of sectioned MRSA upon co-beta-peptide treatment. (a) Untreated control cells of wild type LAC*. (b) LAC* treated with 1×MIC co-beta-peptide. (c) Untreated cells of WTA-deficient mutant LAC*ΔtagO. (d) LAC*ΔtagO treated with 1×MIC co-beta-peptide. (e) Schematics of interaction between co-beta-peptide and cell envelope of MRSA.

Potential of beta-lactam antibiotics against MRSA

Peptidoglycan synthesis in *S. aureus* involves the cooperative functioning of several penicillin-binding-proteins (PBPs). Among the four PBPs that *S. aureus* expresses, PBP2 is most extensively studied and has been shown to be bifunctional: it acts as a transglycosylase to catalyze the linkage of the acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) to elongate the disaccharide strand via glycosyl bonds; it also acts as transpeptidase to catalyze the linkage of the NAM domain with a stem peptide from adjacent peptidoglycan strand to form a crosslinked mesh via pentaglycyl bonds. Beta-lactam antibiotics target the PBPs by binding to the transpeptidase domain to inhibit the proper crosslinking of cell wall in methicillin-sensitive *S. aureus* (MSSA)²⁸⁸. On the other hand, methicillin-resistant *S. aureus* (MRSA) harbours a *mecA* gene to encode an alternative protein PBP2a that possesses transpeptidase function whilst having significantly reduced binding affinity to beta-lactam antibiotics³². PBP2a lacks the function of transglycosylase and requires the cooperative transglycosylase function of PBP2 for proper cell wall synthesis²⁸⁹. However, upon beta-lactam antibiotics treatment (especially those targeting PBP2), the final crosslinking of cell wall is insufficient and requires the cooperative transpeptidase function of PBP4, which is otherwise dispensable in MSSA²⁹⁰. WTA plays an important role in beta-lactam resistance and is intimately

involved in the peptidoglycan synthesis of MRSA. Firstly, WTA serves as a scaffold for PBP2a that is essential for beta lactam resistance²⁷⁸. Secondly, locally synthesized WTA is the signal molecule for the proper recruitment and localization of PBP4²⁹¹. Hence MRSA mutants lacking proper functional WTA are sensitive to beta-lactam antibiotics²⁹². Similarly, antimicrobial agents that disturb WTA synthesis or structural integrity show synergy with beta-lactam antibiotics (especially those targeting PBP2) against MRSA.

Hence, we speculated that the co-beta-peptide could re-sensitize MRSA to beta-lactam antibiotics by targeting WTA. Oxacillin, a beta-lactam antibiotic targeting PBP2, is a first-line therapy for treatment of *Staphylococcal* infection. Checkerboard method was used to study the synergistic effect between co-beta-peptide and oxacillin, where synergy is defined using the fractional inhibitory concentration (FIC) values²⁹³: if $FIC < 0.5$, the combination is synergistic; if $0.5 \leq FIC < 1$, there is partial synergy; if $FIC = 1$, there is only additive effect but no synergy; if $(1 < FIC < 4)$, the combination is indifferent; if $(FIC > 4)$, the combination is antagonistic. A strong partial synergy was observed, where adding co-beta-peptide at $\frac{1}{2} \times MIC$ could effectively potentiate oxacillin by 4- to 128-fold (Table 4-7). The synergistic/strong additive effect of WTA-targeting compounds and beta-lactam antibiotics has been reported for small molecules (*e.g.* tunicamycin²⁷¹, ticlopidine²⁸⁴) and polymers (*e.g.* BPEI²⁷⁹). However, the small molecules could easily select spontaneous escape MRSA mutants with altered WTA synthetic pathways, and their high mutation frequency remains a problem towards clinical usage²⁹⁴. The potential *in vivo* toxicity of cationic polymers such as BPEI also diminishes their biomedical applications. Rather than targeting the highly mutable synthetic pathway of WTA, the co-beta-peptide physically interacts with WTA and

disrupt the integrity of the WTA. The co-beta-peptide, with ultra-low frequency of spontaneous resistance in MRSA and ultra-low *in vivo* toxicity, is a promising candidate for further development as beta-lactam adjuvants.

Table 4-7 Synergistic study of oxacillin and copolymer against HA-MRSA strains.

HA-MRSA strains	MIC of co-beta-peptide	MIC of Oxacillin alone	MIC of oxacillin upon addition of copolymer			FIC
			+1/2 MIC copolymer	+1/4 MIC copolymer	+1/8 MIC copolymer	
ATCC Baa38	16	4	0.125	2	4	0.53
ATCC Baa39	16	8	2	4	8	0.75
ATCC Baa40	16	16	0.25	0.25	8	0.265
ATCC Baa44	16	128	1	128	128	0.5

In summary, we investigated the mechanism of action (MoA) of copolymer using WTA-deficient mutants (LAC* Δ tagO) and showed that WTA is an indispensable target from both the susceptibility test and visualization of sectioned bacterial cells. As expected, the copolymer also potentiated PBP2 targeting beta-lactam antibiotic oxacillin against four hospital-associated (HA-)MRSA, corroborating its WTA-targeting MoA.

Chapter 5 Block co(beta-peptides) eradicate antibiotic-tolerant persisters and biofilm *in vitro* and *in vivo*

Antimicrobial resistance in bacteria is a serious and growing clinical problem, eroding the therapeutic armamentarium and leaving limited treatment options for certain infections. Compounding the difficulty of treating antibiotic resistant strains is the presence of persisters, a subpopulations which is antibiotics-tolerant due to its metabolic inactivity^{71, 295}, and the capacity of bacteria to develop biofilms²⁶, both of which lead to chronic and recurrent infections^{13, 81, 296-297}. *S. aureus* is prone to form biofilms and also exists in the form of metabolically inactive antibiotic-tolerant persister phenotype²⁶. Last resort antibiotics such as vancomycin are largely ineffective against *S. aureus* persisters and biofilms²⁷. New therapeutics are needed to combat the spread of difficult to treat drug-resistant *S. aureus* infections. Alternative antibacterial agents should have bactericidal activity against replicating cells, persisters, and established biofilms. Cationic alpha-peptides and membrane-active agents have been investigated as alternative antimicrobials to combat biofilms and persisters^{91, 191} but unselective toxicity is a complicating factor²⁹⁸.

Besides *Staphylococci*, vancomycin-resistant *Enterococci* is also listed as high priority for R&D of new drugs²⁹⁹. Another important Gram-positive pathogen, *Streptococcus*, is the leading cause of bacterial pneumonia and meningitis in the United States and its threat level is considered serious by Center for Disease Control and Prevention (CDC, USA). The emergence of resistance in these Gram-positive bacteria are particularly worrisome. In addition to the problem of antibiotic-resistance, these Gram-positive bacteria are notorious for their ability to develop biofilms, a 3D structure which serves as a protective shield for biofilm-associated

bacteria and significantly reduces their susceptibility to antimicrobial agents and human immune clearance^{18-19, 300-301}. Though recent developments enable some antimicrobial agents to sufficiently diffuse into biofilms to kill bacteria residing deep inside³⁰²⁻³⁰⁵, this leaves behind the biofilm biomass as substrate for future microbial re-colonization and persistent infection¹⁷⁸. Hence, dispersal of protective extracellular matrix of biofilm is of great interest for agent development. Current biofilm dispersal strategies include mechanical debridement, enzymatic degradation, synthetic- or bio-surfactants, and biofilm modulation with quorum-sensing molecules¹⁸. However, these strategies suffer from limited access to biofilm inside human body, low proteolytic stability, high toxicity and narrow spectrum of targeted types/strains of biofilms¹⁸. Biofilm dispersal strategy effective against all strains of Gram-positive bacteria has rarely been reported¹⁵⁹.

In this Chapter, we demonstrate that, unlike classical antibiotics, **PDGu(7)-b-PBLK(13)** retains potency against MRSA persister cells and biofilms. The block copolymer also effectively removes biofilm biomass but the homocationic beta-peptide (**PBLK(20)**) cannot. Further, the co-beta-peptide could disperse the biofilm of all tested Gram-positive bacteria (five species across two genres) possibly by targeting a universal component on the bacterial envelope – the WTA.

Further, the block copolymer is bactericidal against MRSA in various murine models of systemic acute and established infections, and also in an *ex vivo* human skin infection model, while having no *in vivo* acute toxicity in murine repeated dosing studies. This study opens up new possibilities for treatment for recalcitrant MRSA infections, and sheds light on a broad-spectrum approach for Gram-positive biofilm dispersal by targeting the WTA.

Section 5.1 *In vitro* persister eradication

Persister cells represent a subpopulation of bacterial cells that can survive intensive antibiotic treatment without being genetically resistant. The persister cells can withstand extensive antibiotic treatments and resume growth when antibiotic treatment is ceased⁷². Persister cells are reported to be closely associated with recalcitrant infections such as urinary track infections³⁰⁶. Emergence of resistance could also be promoted by the presence of persister cells, possibly due to prolonged survival of the organism³⁰⁷. Hence, effective eradication of persister cell reservoir is clinically significant to reduce calcitrant infection, prevent re-colonization when antibiotic pressure drops, and diminish antibiotic resistance development.

Since **PDGu(7)-*b*-PBLK(13)** kills *S. aureus* by surface contact-induced membrane/envelope damage, we hypothesized that the block copolymer may retain potency against persisters and *S. aureus* biofilms⁹¹. In this section, we adopted three well-accepted assays to demonstrate efficacy of copolymer against persisters, *i.e.* nutrient-starved, stationary phase, and antibiotic-selected persisters. Since the phenomenon of persister was initially described for antibiotic-sensitive strains⁷², we included the assay for both pen-sensitive *S. aureus* (SA29213) and pen-resistant MRSA USA300.

Kill-kinetics of nutrient-starved persisters

Nutrient-starved persisters were generated by passaging *S. aureus* and MRSA in PBS medium, a condition under which the bacteria can survive for extended periods of time without replicating. Consistent with published literature²⁹⁵, non-replicating MRSA was phenotypic drug resistant to antibiotics from various categories (including vancomycin, oxacillin, rifampicin, *etc.*) up to a dose 100×MIC

(Figure 5-1a). Conversely, **PDGu(7)-b-PBLK(13)** was highly potent against non-replicating starved persister *S. aureus* at a concentration as low as 2-fold its MIC (Figure 5-1b). Similar effect was observed in pen-susceptible *S. aureus* SA29213, where copolymer at 2×MIC showed much superior bactericidal effect (Figure 5-1d) compared with multiple antibiotic controls at 100×MIC (Figure 5-1c).

Figure 5-1 Kill-kinetics of various antibiotics at 100×MIC and **PDGu(7)-b-PBLK(13)** against non-replicating persisters of (a, b) MRSA USA300 and (c, d) SA29213. Antibiotic controls are shown at left panel (a, c) and copolymer shown at right panel (b, d).

Kill-kinetics of stationary phase *S. aureus*

Conlon *et al* reported that stationary phase *S. aureus* exhibit antibiotic tolerance phenotype and they used stationary phase *S. aureus* culture to test

persister-killing efficacy of compounds⁷¹. Following the published protocols^{70-71, 76}, we tested the kill-kinetics of copolymer and various antibiotic controls against stationary phase bacteria (both SA29213 and MRSA USA300). Copolymer at a single concentration of 10×MIC was used to compare with antibiotics at 100×MIC. As expected, the copolymer effectively killed the stationary phase bacteria, whilst antibiotic controls even at 100×MIC could not achieve a similar efficacy. The copolymer completely eradicated 10⁹ CFU/mL of the stationary phase USA300 (Figure 5-2a) and SA29213 (Figure 5-2b) within 5 hours, whilst antibiotics remained ineffective even after 24 hours treatment. These results corroborated well with the nutrient-starved persisters, further highlighted the persister eradication capability of the copolymer irrespective of the persister types.

Figure 5-2 Kill-kinetics of various antibiotics at 100×MIC and **PDGu(7)-b-PBLK(13)** at 10×MIC against stationary phase **(a)** MRSA USA300 and **(b)** SA29213.

Kill-kinetics of antibiotic-induced persisters

Besides Type I persisters that are induced by environmental stresses such as starvation and cell-cell competition in stationary phase, Type II persisters arise spontaneously in the absence of a “trigger”⁸⁰. For example, *S. aureus* in exponential growth phase could stochastically enter persister state due to a reduced ATP production. This pre-existing sub-population of persister cells can be isolated with antibiotic treatment at 10×MIC⁷¹. Hence, a third assay was adopted to select type II persisters using high concentration of antibiotics. The hallmark of persistence is the bimodal (or multimodal) killing curve⁷², as shown in the antibiotic treatment curves (black line) in Figure 5-3. It is interesting to note that, though both MRSA USA300 and SA29213 are susceptible to gentamicin and ciprofloxacin, antibiotics at 10×MIC could not completely eradicate the exponential phase bacteria, leaving behind a sub-population tolerant to antibiotics. **PDGu(7)-b-PBLK(13)** effectively eradicated antibiotic-selected persisters that escaped killing by 10×MIC gentamicin and ciprofloxacin treatment (Figure 5-3).

Figure 5-3 Kill-kinetics of **PDGu(7)-b-PBLK(13)** at 4×MIC against persisters generated by 10×MIC (a, c) gentamicin and (b, d) ciprofloxacin treatment, where (a, b) were MRSA USA300 and (c, d) were SA29213.

Section 5.2 MRSA biofilm bacteria eradication and biomass dispersal

Biofilm bacteria remain as a huge challenge for current antibiotics as they are usually 1,000 times more difficult to treat than planktonic bacteria²². With advances in medical interventions and treatments, biofilm bacterial infection of wounds and medical implants are on the rise. Bacteria could colonize and form biofilms on medical devices such as urinary, arterial and venous catheters and shunts. Biofilm associated infections are also identified with respirators, contact lenses, artificial implants such as pacemakers and heart valves^{300, 308}.

In the subsequent section, we demonstrated that the copolymer effectively eradicated the major types of biofilms formed by CA-MRSA USA300 and various

strains of HA-MRSA; it is also effective against preformed biofilms of methicillin-resistant *Staphylococcus epidermidis* (MRSE).

Eradication of CA-MRSA biofilm

Owing to the versatile genetic pathways, many surface molecules have been identified in the biofilm formation of *S. aureus*⁹³. One of the most commonly reported surface proteins involved in the biofilm of CA-MRSA is fibronectin-binding protein (FnBP), a cell-wall anchored biofilm-associated protein. In the first study, we developed preformed biofilm of CA-MRSA USA300 maintained in a broth medium supplemented with glucose, a condition known to form FnBP type of biofilm³⁰⁹. To test the biofilm eradication efficacy of the copolymer, we adopted the MBEC™ protocol (previously known as Calgary biofilm assay)³¹⁰. The biofilm was established on the pegs of MBEC™ and a graphic illustration of the testing procedures were shown below (Figure 5-4).

Figure 5-4-1 A schematic illustration of the setup of MBEC™ plate. The pegs are attached on the lid (cover of the plate) and immersed into the bacteria suspension in the 96-well plate. Biofilms can be developed on the pegs after prolonged incubation under appropriate conditions.

Figure 5-4-2. A schematic illustration of the MBEC test procedure. Preformed biofilms were established in the pegs and exposed to antimicrobial compounds. To determine bacteria CFU on each peg after challenging with compounds, the bacteria in biofilm were dislodged into a new 96-well plate by sonication, followed by serial-dilution and plating onto agar plates.

The copolymer **PDGu(7)-*b*-PBLK(13)** was effective at eradicating preformed MRSA biofilms and greatly outperformed vancomycin, which had an insignificant effect on biofilm bacteria (Figure 5-5a). **PDGu(7)-*b*-PBLK(13)** has the ability to kill bacteria residing in biofilm and significantly reduces viable bacteria at a concentration of 32 $\mu\text{g/mL}$, achieving log reduction of 2.99 (99.9% killing). Vancomycin, the last resort drug against MRSA, showed minimal killing effect ($<0.5 \log_{10}$ reduction) against biofilm bacteria even at a concentration as high as 256 $\mu\text{g/mL}$. Compared to copolymer, homocationic **PBLK(20)** was equally effective in killing the biofilm bacteria but its toxicity was a significant drawback.

In addition to killing bacteria in biofilm, the block copolymer effectively dispersed the biofilm itself. The untreated peg surface was heavily colonized by MRSA USA300, which formed dense biofilm with complex 3D structure (Figure 5-5b I). On the contrary, peg treated with 64 $\mu\text{g/mL}$ copolymer showed significantly

reduced biofilm, leaving behind a clean substrate with minimal bacteria (Figure 5-5b II).

Time-lapse Confocal was used to reveal the kinetics of biofilm dispersal. 24-hour preformed biofilms were established in collagen-coated petri-dish, and treated with either copolymer (Figure 5-5c I) or homocationic (Figure 5-5c II) at 32 $\mu\text{g/mL}$. Biofilms were stained with Live/Dead BacLight™ kit (green SYTO 9 and red propidium iodide) to facilitate visualization. Besides staining biofilm bacteria, SYTO 9 also stains the biofilm matrix component such as eDNA³¹¹. The copolymer showed time-dependent dispersal of biofilm biomass, and killed the bacteria inside biofilm (as shown by the propidium iodide red staining of the bacteria). After 3 hours, the biofilm bacteria and matrix were almost completely dispersed (Figure 5-5c I). However, the homocationic **PBLK(20)** could not disperse the biofilm biomass, and the dead bacteria remained in the biofilm even after prolonged period of time (Figure 5-5c II). The complete time points of the kinetic study were shown in Figure 5-6.

Figure 5-5 (a) Activity of **PDGu(7)-b-PBLK(13)** and **PBLK(20)** on established MRSA USA300 biofilms using the MBECTM Assay. Data are presented as mean ± standard deviation of three independent experiments. (b) FESEM image of MBECTM microtiter plate pegs: (I) control peg without treatment and (II) peg treated with **PDGu(7)-b-PBLK(13)**. (c) Confocal microscopy image of (I) **PDGu(7)-b-PBLK(13)** and (II) **PBLK(20)** treated MRSA USA300 biofilm at t = 0 min, 30 min and 3 hours. Biofilms were stained with Live/Dead BacLightTM kit.

Figure 5-6 Time lapse confocal images of biofilm stained with Live/Dead BacLight dye. (a) Treated with 32 μ g/mL **PBLK(20)** and (b) 32 μ g/mL **PDGu(7)-b-PBLK(13)**. Z stack thickness = 10 μ m.

Bacteria released from biofilm during dispersal phase is particularly problematic. These bacteria are not only the source for acute infection in the host and transmission of infection between hosts, they also exert enhanced virulence such as increased killing of macrophage¹⁶⁶. Hence, we further investigated the viability of bacteria dispersed by the copolymer. Mature biofilms were established on petri-

dishes as previously described, and treated with PBS (control) or 32 μ g/mL **PDGu(7)-b-PBLK(13)**. After 3-hour treatment, treated biofilm were imaged using Confocal microscopy (Figure 5-7); the supernatant which contained dispersed bacteria were removed from the petri dish and imaged separately (Figure 5-8). Biofilm treated with PBS control remained intact (Figure 5-7 I and II), and minimal live bacteria was released from biofilm (Figure 5-8 I and II). For copolymer treated biofilm, only minimal dead bacteria remained in the biofilm (as indicated by the staining of propidium iodide), and the biofilm was substantially dispersed (Figure 5-7 III and IV). The copolymer-dispersed bacteria from biofilm was also dead (Figure 5-8 III and IV), which reduced the risk of infection dissemination to other organs or septic shock from a sudden release of live bacteria from biofilm; it kills the biofilm bacteria in addition to dispersing them.

Figure 5-7 MRSA USA300 biofilm bacteria on collagen-coated glass bottom petri dish (I, II) untreated control, (III, IV) copolymer treated sample. Bacteria stained with Live/Dead BacLight™ dye. (I, II) PBS control with minimal bacteria dispersed from biofilm and biofilm bacteria remain alive; (III, IV) Dispersed biofilm after treatment with 32 μ g/mL **PDGu(7)-b-PBLK(13)** in PBS for 3 hours. Biofilm on petri dish was dispersed and the minimal remaining Bacteria are dead, indicated by PI staining.

Figure 5-8 Supernatant taken from the biofilm of Figure 5-7 (I, II) untreated control, (III, IV) copolymer treated sample. Bacteria stained with Live/Dead BacLight™ dye. (I, II) PBS control with minimal bacteria dispersed into supernatant and bacteria remain alive; (III, IV) Dispersed biofilm bacteria after treatment with 32μg/mL **PDGu(7)-b-PBLK(13)** in PBS for 3 hours. Majority of bacteria released from biofilm into supernatant are dead, indicated by PI staining.

Eradication of HA-MRSA and MRSE biofilms

(CA-)MRSA USA300 maintained in a broth medium supplemented with glucose typically forms biofilm involving cell-wall anchored protein (FnBP)³⁰⁹, whilst many (HA-)MRSA³¹²⁻³¹³ and *S. epidermidis*^{64, 314} strains form biofilms involving the polysaccharide intercellular adhesin encoded by the *ica* locus³¹⁵. To determine if the block copolymer is active against other types of biofilms, biofilms formed by various HA-MRSA and methicillin resistant *S. epidermidis* (MRSE) strains under conditions promoting the *ica* locus expression¹¹⁸ were treated with the copolymer. We tested our copolymer against 6 new HA-MRSA/MRSE strains, specifically MRSA (**a1**) ATCC BAA38, (**a2**) ATCC BAA39, (**a3**) ATCC BAA40, and (**a4**) ATCC BAA44, and MRSE (**b1**) ATCC 35984, and (**b2**) ATCC 700563. The copolymer **PDGu(7)-b-PBLK(13)** was more active than vancomycin in eradicating the biofilms of HA-MRSA and MRSE strains (Figure 5-9). Hence, our

copolymer is effective not only against MRSA biofilms involving fibronectin-binding protein, but also against other major types of biofilm formed by HA-MRSA and MRSE.

Figure 5-9 PDGu(7)-b-PBLK(13) shows dose-dependent eradication of biofilm bacteria ((a) HA-MRSA and (b) MRSE) under conditions that promote polysaccharide intercellular adhesion; y-axis: biofilm bacteria (CFU/mm² peg) formed by HA-MRSA (a1) ATCC BAA38, (a2) ATCC BAA39, (a3) ATCC BAA40, (a4) ATCC BAA44 and MRSE (b1) ATCC 35984, (b2) ATCC 700563. (Vancomycin is used as antibiotic control.) Data are presented as mean $\pm$ standard deviation.

For a short summary, we show that the block copolymer can eradicate the persister and biofilms of clinically relevant community-acquired MRSA (USA 300). We also showed that our copolymer is just as effective against biofilms of HA-MRSA (and MRSE) strains with resistance to multiple conventional antibiotics. Eradication of persisters and biofilms remains one of the biggest challenges in antibacterial drug discovery. Antibiotic-tolerant bacteria are associated with longer treatment time and relapse of infection. Because most antibiotics target macromolecular machinery only essential for active replication, they are significantly less potent against non-replicating persisters or established biofilms. **PDGu(7)-b-PBLK(13)** kills replicating, antibiotic-tolerant persisters, and biofilm-associated MRSA, both *in vitro* and *in vivo*. The ability of our co-beta-peptide to kill all the sub-populations (planktonic, persister and biofilm states) of MRSA bacteria is attributable to its mechanism(s) of kill -- membrane disruption and interface weakening effects which are not related to metabolism. The reduced tendency of the block copolymer to bind mammalian membranes is linked to their less negatively charged surface^{54, 316}.

Upon surface-contact with bacterial membrane, the cationic block undergoes transition from a random coil in free solution to a helix to expose cationic charges. The block copolymer possesses a unique bacteria-triggered surfactant effect – the cationic block adsorbs onto the negatively charged bacterial envelope while the hydrophilic sugar block has a strong tendency to promote dissolution, resulting in a “surfactant-like” solvation of bacteria from biofilm. The amine group of the cationic block dominates the interactive topology with erythrocytes but its hydrophilicity minimizes hemolysis. Further, the neutral sugar block also increases the hydrophilicity of the block copolymer. Other antimicrobial peptides and

biosurfactants (such as surfactin, rhamnolipid or phenol-soluble modulins) are intrinsically amphiphilic with exposed hydrophobic domains in free solution and are typically hemolytic since their freely exposed hydrophobic moieties would interact with erythrocytes¹⁵⁷.

Biofilm eradication using conventional antibiotics is typically challenging^{178, 317}. The limited efficacy of vancomycin against biofilms is not an exception; many other antibiotics that are commonly used for MRSA infection have significantly reduced efficacy against biofilm bacteria³¹⁸. Besides antibiotics, our copolymer outperforms many cationic antimicrobial peptides and conventional antiseptic therapeutics in established wound infections that have been previously reported^{191, 319}, not to mention its superior safety profile that makes it suitable for translation into clinics.

The copolymer eradicates biofilm MRSA and also disperses the biomass. Since the block copolymer forms an anti-fouling **PDGu** coating around the bacterial cell envelope, the adhesion of the bacteria to extracellular polymeric substances (EPS) and to substrates is reduced, explaining the strong dispersing effect of **PDGu(7)-b-PBLK(13)** on biofilms (Figure 5-6). The coated bacteria can effectively detach due to reduced surface hydrophobicity and interaction with biofilm matrix, leading to biofilm dispersal^{159, 320-321}. Moreover, the copolymer biofilm eradication effect is observed in the major types of biofilms formed by different MRSA (and MRSE) strains under various conditions, which include the types involving fibronectin binding protein as well as polysaccharide intercellular adhesin. This is clinically significant since MRSA is a common pathogen forming biofilms during infection, as well as on medical devices^{243, 322}.

Section 5.3 Broad-spectrum Gram-positive bacteria biofilm dispersal

In previous session, we have proved that the co-beta-peptide “coats” a hydrophilic non-fouling layer on MRSA bacteria surface, leading to reduced interaction with biofilm matrix and eventually biofilm dispersal. Since WTA is a generic component present on the surface of Gram-positive bacteria²⁷¹, we hypothesized that the co-beta-peptide could also indiscriminately target the WTA on other Gram-positive bacteria, enabling a broad-spectrum biofilm dispersal effect.

Besides MRSA and methicillin-resistant *Staphylococcus epidermidis* (MRSE) biofilms that have been proven previously, in this study we showed that the co-beta-peptide disperses pre-formed biofilms of 5 clinically significant Gram-positive bacteria (*E. faecalis* and 4 *Streptococci*), possibly due to its bacteria-responsive surfactant effect – the cationic block interacts with WTA on bacteria surface and the non-fouling glycosylated block reduces physical interaction between bacteria and biofilm matrix, eventually leading to detachment of bacteria and dispersal of biofilm matrix. This study reinforces the significance of WTA in developing antimicrobial agents and sheds light on a new strategy – targeting of WTA – for dispersal of Gram-positive bacterial biofilms.

We tested its preformed biofilm dispersal effect against a panel of Gram-positive bacteria: *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Streptococcus pneumonia*, *Streptococcus mutans* and *Enterococcus faecalis*. The co-beta-peptide showed dose-dependent biofilm dispersal for all tested pathogens, and the efficacy was significantly superior to that of the control antibiotic/antiseptic at most concentrations (Figure 5-10).

Figure 5-10 PDGu(7)-b-PBLK(13) (purple) shows dispersal of biofilm biomass of different Gram-positive bacteria: **(a)** *Streptococcus agalactiae* ATCC 13813, **(b)** *Streptococcus pyogenes* 12344, **(c)** *Streptococcus pneumonia* 6303, **(d)** *Streptococcus mutans* 10449 and **(e)** *Enterococcus faecalis* V583. The control antibiotic/antiseptic control (grey) is ineffective in the biomass removal. Biomass is quantified by crystal violet staining.

Biofilm dispersal using both synthetic and bio-surfactants has been reported. Compared with other dispersal agents such as enzyme or Quorum-sensing molecules, surfactants such as Polyhexamethylene biguanide or Chlorhexidine are particularly interesting due to their stability, rapid action and low cost of production³²³. However, the poor selectivity and strong hemolytic activity are major drawbacks. The double hydrophilic block co-beta-peptide, on the other hand, is highly biocompatible and non-hemolytic. Unlike conventional surfactants which have distinct hydrophobic and hydrophilic domains, the co-beta-peptide belongs to a new and emerging field: double hydrophilic stimulus-responsive surfactants. In aqueous environment, the co-beta-peptide remains as true solution, not forming supramolecular structures, in contrast to surfactant with well-defined micro/nanostructures. Upon interaction with Gram-positive bacteria, cationic block interacts strongly with WTA on bacterial cell envelope (the substrate) and the glycosylated block remains dissolved, forming what could be described as a “cellular scale amphiphilic complex” – with a “corona” formed by the hydrophilic glycosylated blocks of the copolymer molecules, which densely cover the cell surface; the “core” is the “hydrophobic” cationic blocks-bacteria complex. The antifouling layer then enables detachment of bacteria from the biofilm matrix and the dispersal of the biofilm due to reduced hydrophobic interactions with extracellular matrix.

Dispersal of biofilms formed by the various Gram-positive strains, across three genres (*Staphylococci*, *Streptococci* and *Enterococci*), is a clinically significant result. *Staphylococcus aureus* is the most common pathogen associated with indwelling medical devices, which is attributable to its biofilm-forming ability³²⁴. *Staphylococcus epidermidis* forms biofilms on implants and causes

infections with indwelling biomaterials³²⁵. *Streptococcus agalactiae*, group B *streptococcus* (GBS), forms biofilm in vaginas and recta of pregnant women and is a leading cause of neonatal meningitis³²⁶. *Streptococcus pyogenes*, group A *streptococcus* (GAS), forms biofilms on the skin and pharynx and causes illnesses ranging from impetigo and pharyngitis to life-threatening toxic shock syndrome³²⁷. *Streptococcus pneumoniae* forms biofilms in nasal cavity and lungs, and can disseminate to cause otitis media, pneumonia and meningitis³²⁸. *Streptococcus mutans* forms biofilms on tooth surfaces, producing acidified microenvironment and leading to dental caries³²⁹. *Enterococcus faecalis* biofilms are involved in endocarditis, urinary tract and wound infections; the bacteria can also disseminate to cause environmental and food contamination³³⁰. Broad-spectrum biofilm dispersal against multiple Gram-positive genuses is particularly attractive, since many biofilm-associated infections are polymicrobial, and targeting only one specific pathogen becomes less effective³⁰.

Figure 5-11 Schematic illustration of MRSA bacteria eradication and biofilm dispersal effect of the cationic block copolymer **PDGu(7)-b-PBLK(13)**. The copolymer binds to bacterial cell envelope, coating an anti-fouling layer on bacterial cells, leading to cell death and biofilm dispersal.

Section 5.4 *In vivo* biocompatibility and antimicrobial properties

An ideal antibacterial would combine excellent biocompatibility and bactericidal activity, both *in vitro* and *in vivo*. In this section of *in vivo* studies, we examined the biocompatibility in mice, antimicrobial efficacy in murine acute infection models, murine established biofilm models and *ex vivo* human skin biofilm infection model.

In vivo biocompatibility of PDGu(7)-*b*-PBLK(13)

Since the major drawback of cationic antimicrobial peptides/polymers are the potential toxicity, we first evaluated the acute toxicity of the block copolymer in mice. A repetitive dosing scheme was adopted, with a daily intravenous (i.v.) injection of **PDGu(7)-*b*-PBLK(13)** at 10 mg/kg for up to 7 days. Mice body weight, blood biomarkers were monitored throughout the course of injection. Copolymer at a cumulative dose of 70 mg/kg (10 mg/kg-day $\times$ 7days) was well tolerated in all mice, with no death observed up to 7 days post last injection (Figure 5-12a). As a comparison, we also conducted the i.v. injection of homocationic **PBLK(20)** at 10 mg/kg in mice. However, 1 out of 6 mice died within 1-day after a single injection, indicating the much higher toxicity of **PBLK(20)**. These data corroborated with the *in vitro* biocompatibility test, where incorporation of **DGu** sugar block could significantly reduce toxicity.

Alanine transaminase (ALT) level, aspartate transaminase (AST) level and their ratio (AST/ALT) are clinically significant biomarkers for hepatotoxicity. AST and ALT are enzymes normally contained in liver cells. Upon liver damage, the enzymes are released into blood and an increase of the enzyme level can be directly

related to the extent of tissue damage²²⁹. **PDGu(7)-b-PBLK(13)** induced no liver toxicity, confirming its low *in vivo* acute toxicity (Figure 5-12b).

Figure 5-12 In vivo repetitive toxicity of daily 10 mg/kg i.v. injection of **PDGu(7)-b-PBLK(13)** for 7 consecutive days. **(a)** Mice weight (left y-axis) and cumulative dosage (right y-axis) over 14 days. **(b)** ALT and AST biomarker changes at t = 0 and 7 days.

Besides ALT and AST, other biomarkers were also measured as shown in Table 5-1. TBIL (total bilirubin) measures the total amount of bilirubin (both conjugated and unconjugated with sugar) in the serum. Bilirubin is a catalytic product during the destruction of aged red blood cells, and it is processed by the liver to be eliminated from the body. Possible causes of high TBIL include liver damage and elevated red blood cell hemolysis²³⁰. Serum creatinine (CRE) is a muscle metabolic byproduct and is cleared in the kidney. Elevated creatinine level is an indicator of kidney damage that leads to reduced clearance of creatinine. Blood glucose (GLU), blood urea nitrogen (BUN), potassium and sodium ion concentrations are other common biomarkers for kidney function and the electrolyte balance in the blood. Abnormalities of Total protein (TP), Albumin (ALB) and Globulin (GLO) are indication of liver, kidney or immune system disorder. No

statically significant changes were induced by **PDGu(7)-b-PBLK(13)** in all the biomarkers, confirming its low *in vivo* acute toxicity (Table 5-1).

We further conducted histopathology studies of the major organs (kidney, liver and spleen) of copolymer treated mice (Figure 5-13). The sections were examined by a pathologist and no pathological changes were detected in tissue sections of treated mice compared with that of the controls. These results further confirmed that a repetitive dosage of copolymer has negligible acute tissue toxicity.

Figure 5-13 (a to c) Histology of organs of untreated healthy (left) and **PDGu(7)-b-PBLK(13)** treated (right) mice. **(a)** Kidney **(b)** Liver **(c)** Spleen. Copolymer injected at 10 mg/kg daily for 7 days.

Table 5-1 Blood biomarkers of mice before injection, 1-day, 4-day and 7-day post first injection. **PDGu(7)-b-PBLK(13)** injected at 10 mg/kg daily for 7 days.

Biomarkers	10 mg/kg daily										
	t=0 day		t=1 day			t=4 day			t=7 day		
	Mean	SD	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value
TP	50.98	1.32	49.74	2.37	0.6035	51.76	3.67	0.6954	51.66	2.05	0.5238
ALB	29.42	2.58	28.34	2.21	0.8766	29.06	5.44	0.9129	29.34	2.12	0.8889
GLO	21.56	1.65	21.40	1.31	0.3588	22.70	1.82	0.4349	22.32	1.81	0.6905
TBIL	2.89	0.90	3.19	0.65	0.4717	2.40	0.21	0.3633	3.342	1.06	0.5476
ALT	44.80	6.57	44.00	7.04	0.6037	40.80	4.92	0.4118	42.8	5.26	0.5476
AST	75.60	13.01	75.40	7.37	0.6929	81.80	11.95	0.3036	71.6	9.40	0.6905
BUN	9.43	2.05	9.72	0.46	0.2214	9.05	0.67	0.6773	9.504	0.86	0.7857
CRE	55.20	13.22	61.60	7.70	0.6545	58.40	14.96	0.7927	55.6	22.23	0.8413
GLU	7.94	0.89	7.24	0.55	0.2391	6.38	0.41	0.0625	7.816	1.02	>0.9999
Na+	6.57	0.62	6.13	0.94	0.5808	6.48	0.51	>0.9999	5.986	0.37	0.0952
K+	142.00	4.74	139.60	11.24	0.0594	140.80	3.56	0.7500	145.2	4.02	0.3571

***In vivo* efficacy of PDGu(7)-*b*-PBLK(13) acute infection models**

The *in vivo* efficacy of **PDGu(7)-*b*-PBLK(13)** was first evaluated in a mouse model of acute systemic infection. We first conducted the optimization experiment with a range of bacteria concentrations and identified 10^8 CFU/mL of MRSA USA300 as the lethal dose (100% death within 24 hours in untreated mice). Mice were then infected with the lethal dose of bacteria; at 2h post infection, a single 5 mg/kg dose of intraperitoneally (i.p.) injected copolymer resulted in significantly reduced bacterial loads in major organs (Figure 5-14 a to d). Copolymer treatment also led to 100% rescue of the mice (6/6 mice, Figure 5-14e). In contrast, vancomycin treatment at the same dosage achieved only 67% survival (4/6 mice).

Figure 5-14 In vivo efficacy test in murine sepsis model. Vehicle alone (–), vancomycin and **PDGu(7)-b-PBLK(13)** at 5 mg/kg were injected i.p. and bacteria loads were expressed as log CFU of (a) liver, (b) IP fluid, (c) kidney and (d) spleen. (e) Survival% of vancomycin and **PDGu(7)-b-PBLK(13)** treatment.

Besides sepsis model, we also demonstrated the efficacy of copolymer in acute wound infection model. Treatment was applied at 4 hours post infection on a murine excisional wound. **PDGu(7)-b-PBLK(13)** treatment achieved a reduction in bacterial load of four order of magnitude, efficacy that was comparable to vancomycin (Figure 5-15).

Figure 5-15 In vivo efficacy test in murine excisional wound acute infection model. Vehicle alone (–), **PDGu(7)-b-PBLK(13)** or vancomycin at 2.5 mg/kg were applied at a single dose. Dotted line represents limit of detection (25 CFU/wound).

In summary, the copolymer exhibited equivalent (or slightly better) efficacy compared with vancomycin to reduce bacterial loads in acute infection sepsis and wound models. As vancomycin-intermediate and -resistant *S. aureus* (VISA and VRSA) strains are identified with increasing frequency in clinical practice, the need

for alternative anti-*Staphylococcal* agents to treat VISA/VRSA infections has become more apparent³³¹.

***In vivo* efficacy of PDGu(7)-b-PBLK(13) in established infection models**

We progressed to test copolymer efficacy in an established murine excision wound model by initiating treatment 24 hours post infection, by which time the bacteria have developed stable biofilms³³²⁻³³³. In this model, the copolymer treatment achieved a 99.96% reduction in bacterial load, which was significantly better compared to vancomycin (Figure 5-16a). We further progressed to a tougher model involving severely established biofilms. A very mature biofilm was established in the wound with a 72-hour infection duration. Copolymer treatment achieved 99.87% (2.9 log₁₀) reduction in bacterial load, which was significantly better than vancomycin (83.8%, *i.e.* 0.8 log₁₀, reduction) (Figure 5-16b), showing that the block copolymer has high activity even against an established *S. aureus* infection known to be recalcitrant to antibiotic treatment. Besides antibiotics, our copolymer also outperforms many cationic antimicrobial peptides and conventional antiseptic therapeutics in established wound infections that have been previously reported^{191, 319}, not to mention its superior safety profile that makes it suitable for translation into clinics.

Figure 5-16 In vivo antimicrobial activity of **PDGu(7)-b-PBLK(13)** against MRSA USA300 in established murine excision wound **(a)** 24-hour biofilm and **(b)** 72-hour biofilm model. Vehicle alone (–), **PDGu(7)-b-PBLK(13)** or vancomycin at 2.5 mg/kg were applied at **(a)** three times at 4-hours intervals or **(b)** six times over 2 days. ** $p \leq 0.01$, **** $p \leq 0.0001$ by one-way ANOVA followed by Dunnett test.

We further evaluated the efficacy of the copolymer against persisters/biofilm with a deep-seated thigh infection model in neutropenic mice known to be particularly resistant to antibiotic treatment^{91, 334}. In this model, first treatment was applied 24-hour post infection at 20 mg/kg, with a second dose at 20 mg/kg applied 3 hours later. The copolymer achieved a 93.7% (1.2 log₁₀) reduction in bacteria load, whereas vancomycin was completely ineffective (Figure 5-17). The efficacy of the copolymer is superior to the compound (analogue 2, a retinoid derivative) reported recently in *Nature*, which only achieved 85% (0.6 log₁₀) reduction in the same thigh infection model, at a much higher dosage over a longer period of treatment (80 mg/kg every 12 hours for 3 days)⁹¹.

Figure 5-17 *In vivo* antimicrobial activity of **PDGu(7)-b-PBLK(13)** against MRSA USA300 in a deep-seated neutropenic thigh infection model. First treatment was applied 24-hour post infection at 20 mg/kg, with a second dose at 20 mg/kg applied 3 hours later. ** $p \leq 0.01$, *** $p \leq 0.001$ by one-way ANOVA followed by Dunnett test.

***Ex vivo* efficacy of PDGu(7)-b-PBLK(13) in an established wounded human skin model**

In addition to the murine models, we also demonstrated the efficacy of the copolymer with an *ex vivo* human skin model with severely established (48h) infection (Figure 5-18). The copolymer treatment achieved 99.998% ($4.6 \log_{10}$) reduction of bacterial burden in contrast to the 97.3% ($1.6 \log_{10}$) reduction of vancomycin treatment. Copolymer treated *ex vivo* human wound sites were also clear of pus/debris corroborating its antifouling/biofilm dispersal properties (Figure 5-5).

Figure 5-18 *Ex vivo* antimicrobial activity of **PDGu(7)-b-PBLK(13)** against MRSA USA300 in an established wounded human skin model. **(a)** Vehicle alone (—), **PDGu(7)-b-PBLK(13)** or vancomycin control at 100 μ g were applied three times with 3-hours interval between treatments, starting 48-hours post infection. ** $p \leq 0.01$, **** $p \leq 0.0001$ by one-way ANOVA followed by Dunnett test. **(b)** A representative set of wound images by end of the treatment. From left to right: Vehicle alone, vancomycin and copolymer treated samples.

In summary, the new block co-beta-peptide (**PDGu(7)-b-PBLK(13)**) demonstrates excellent bactericidal efficacy against all MRSA sub-populations, *i.e.* replicating, biofilm-associated and antibiotic-induced persister bacteria. It is active against CA-MRSA (USA300) and numerous other MDR HA-MRSA. Its antibacterial activity in established MRSA murine infection models is superior to that of vancomycin, and it exhibits no acute *in vivo* toxicity in repeated dosing

studies at levels above those required for therapeutic efficacy. Further, the copolymer effectively eradicates established MRSA infections in an *ex vivo* human skin model. It also kills biofilm bacteria while effectively dispersing the biofilm mass of CA-MRSA; it also shows efficacy against the major types of biofilms formed by HA-MRSA. The cationic block co-beta-peptide undergoes a bacterial-membrane-triggered conformation change from a random coil to a helix, where it acts as a bacteria-triggered surfactant leading to biofilm dispersal. As resistance towards all classes of antibiotics rapidly evolves and spreads, the outstanding efficacy of **PDGu(7)-b-PBLK(13)** against *S. aureus* persists and biofilms as well as its excellent safety window, makes this block co-beta-peptide a valuable candidate for novel strategies to treat MRSA infections.

Chapter 6 Conclusions and future directions

Section 6.1 Conclusions

In this thesis, we have presented a new enantiomeric block co-beta-peptide, poly(amido-D-glucose)-*block*-poly(beta-L-lysine)) (**PDGu(7)-b-PBLK(13)**) as a promising antibacterial and antibiofilm agent. The new block co-beta-peptide demonstrates excellent bactericidal efficacy against all MRSA sub-populations, *i.e.* replicating, biofilm-associated and antibiotic-tolerant persister bacteria. It is active against community-acquired (CA-)MRSA (USA300) and numerous other multi-drug-resistant (MDR) hospital-associated (HA-)MRSA. Its antibacterial activity in established MRSA murine infection models is superior to that of vancomycin, and it exhibits no acute *in vivo* toxicity in repeated dosing studies at levels above those required for therapeutic efficacy. Further, the copolymer effectively eradicates established MRSA infections in an *ex vivo* human skin model. It kills biofilm bacteria while effectively dispersing the biofilm mass of CA-MRSA; it also shows efficacy against the major types of biofilms formed by HA-MRSA. The cationic block co-beta-peptide undergoes a bacterial-membrane-triggered conformation change from a random coil to a helix, where it acts as a bacteria-triggered surfactant leading to biofilm dispersal. As resistance towards all classes of antibiotics rapidly evolves and spreads, the outstanding efficacy of **PDGu(7)-b-PBLK(13)** against *S. aureus* persists and biofilms as well as its excellent safety window, makes this block co-beta-peptide a valuable candidate for novel strategies to treat MRSA infections.

This co-beta-peptide also exhibited broad-spectrum biofilm dispersal activity by targeting wall teichoic acid (WTA) on the surface of Gram-positive bacteria. We have shown that WTA is an indispensable target for the mechanism of

action (MoA) of the co-beta-peptide. The susceptibility of WTA-deficient mutant LAC* Δ tagO is significantly (16-fold) reduced compared with wild type LAC*. Transmission Electron Microscopy (TEM) of sectioned MRSA cells suggests different MoAs in wild type and WTA-deficient mutants. Corroborating its WTA-targeting ability, the co-beta-peptide potentiated PBP2-targeting beta-lactam antibiotic oxacillin against 4 HA-MRSA strains. Furthermore, the co-beta-peptide dispersed pre-formed biofilms of 5 Gram-positive bacteria, possibly due to its bacteria-responsive surfactant effect – the cationic block interacts with WTA on bacteria surface and glycosylated block acts as a non-fouling corona to reduce physical interaction between bacteria and matrix, eventually leading to detachment of bacteria and dispersal of biofilm matrix. Taken together, this study sheds light on a new strategy in Gram-positive bacteria biofilm dispersal by design of a dual functional co-beta-peptide: a cationic block targeting WTA of Gram-positive bacteria and an antifouling glycosylated block to promote cell detachment from biofilm matrix.

Section 6.2 Future directions

6.2.1 In-depth study of the WTA-targeting MoA

In Chapter 4, we have identified WTA as an indispensable target. However, the in-depth analysis of interaction between copolymer and WTA remains to be elucidated. Three additional assays are proposed to further elucidate the MoA. First, a competitive binding assay will be adopted to study the binding affinity difference of copolymer to wild type and WTA-deficient mutant. Technique such as flow cytometry can be used to quantify the association of rhodamine-labelled copolymer with bacterial cells. Second, besides genetically modified WTA-deficient mutants,

chemical suppression screen is becoming a popular approach to identify molecules targeting WTAs. A few high-throughput screening assays have been reported and two promising WTA-targeting molecules have been identified with *in vivo* efficacy²⁸²⁻²⁸³. Early-stage WTA synthesis inhibitor, tunicamycin, can be used to chemically abrogate WTA to independently prove WTA is involved in the MoA of copolymer. Thirdly, WTA can be extracted from a large volume of cells following published protocol³³⁵. Techniques such as ITC can be used to study the driving force between copolymer and WTA in molecular level. (This technique has been widely adopted in probing the binding mechanism between Lipopolysaccharides (LPS) and LPS-targeting agents³³⁶⁻³³⁷).

6.2.2 Broad-spectrum biofilm dispersal strategy

The broad-spectrum biofilm dispersal effect of the copolymer due to its WTA-targeting and bacteria-induced surfactant effect is inspiring: it sheds light on a new strategy in biofilm dispersal by targeting a universal molecule present on the outermost surface of the bacteria. However, the copolymer has limited killing efficacy against *Streptococci* and *Enterococci* ($\text{MIC} \geq 128 \mu\text{g/mL}$), and dispersal of biofilm could lead to a septic shock due to sudden release of live bacteria. To solve this problem, the copolymer and antibiotic could be used synergistically, where copolymer disperse biofilm and release bacteria from biofilm-state to planktonic state; antibiotic then kills the planktonic state bacteria much more efficiently.

Moreover, the biofilm dispersal effect of the copolymer is currently limited to Gram-positive bacteria. The global priority pathogen list published by WHO has ranked the following carbapenem-resistant Gram-negative bacteria as critical with highest priority for future R&D⁸: *Acinetobacter baumannii*, *Pseudomonas*

aeruginosa and *Enterobacteriaceae* including *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter spp.* New strategies to eradicate Gram-negative bacteria biofilm is urgently needed. Unfortunately, the copolymer failed to disperse biofilms of Gram-negative bacteria, possibly due to its weak interaction with their outer membrane. Similar to WTA in Gram-positive bacteria, LPS abundantly presents on the outer surface of Gram-negative bacteria. LPS has been reported as a trigger to release antimicrobial drug as a result of competitive displacement of polymer³³⁸, and LPS-targeting peptides have been identified³³⁹⁻³⁴⁰. It is expected that incorporation of LPS-targeting segments, in replacement of the WTA-targeting **PBLK** block, to the copolymer could promote binding to Gram-negative bacteria surface, and potentially trigger a similar mechanism – bacteria induced surfactant effect, leading to dispersal of Gram-negative biofilm.

6.2.3 Arginine-based vs lysine-based antimicrobial beta-peptide

Charged amino acids such as arginine and lysine play important roles in biological activities including protein folding³⁴¹ and helix aggregation³⁴². Their interactions with phospholipid bilayers are essential for cell-penetrating peptides (CPPs)³⁴³ and antimicrobial peptides (AMPs)³⁴⁴. AMPs commonly contain charged amino acids of lysine and arginine that are known to demonstrate membrane active properties^{59, 345}. Compared to lysine, arginine is of considerable interest for the development of new antimicrobial peptides/polymers due to its unique properties. Arginine has a higher aqueous pKa of 12–13.7³⁴⁶ and a smaller pKa shift when it is near/at biological membranes. This makes arginine the only amino acid that can maintain positive charge of the side chain even in the hostile membrane environment, to enable more stable electrostatic interaction with anionic components in bacteria

membranes³⁴⁷. Its guanidinium side chain also provides extensive hydrogen bonding with the phosphate head groups of bacterial membrane lipids³⁴⁸. It allows binding in three possible directions to form a larger number of interactions. As such, arginine induces greater membrane deformation than lysine by attracting more water or lipid head groups with multiple H-bonds due to its geometry and hydrogen bonding capability³⁴⁷. Many reported AMP design favors arginine over lysine due to its superior antimicrobial efficacy and selectivity³⁴⁸⁻³⁵². Besides the bactericidal property, arginine is also reported to exert biofilm modulating property in biofilm-forming oral bacteria³⁵³⁻³⁵⁴ and anti-biofilm property in pathogenic bacteria³⁵⁵. The promising antimicrobial activity makes arginine and its derivatives attractive for further development. We are developing arginine-based block co-beta-peptide to investigate the antimicrobial and anti-biofilm properties.

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Appendix 1 Synthetic scheme and NMR characterization of ruthenocenecarbonyl chloride

Experimental Procedure:

Ruthenocene (1 g) was dissolved in 50 mL dichloromethane, and cooled to -5 °C with salt-ice bath. Then, 1.3 mL 2-chlorobenzoyl chloride (900 mg) was added and stirred at that temperature for 10 min under N_2 . After the solution was chilled thoroughly, solid anhydrous aluminum chloride (850 mg) was added in small portions at a rate so that the reaction mixture temperature did not exceed 0 °C. The solution was stirred for 60 minutes at that temperature and then another 16 h at room temperature. The reaction mixture was cooled to 0 °C. Then 80 mL ice water was added cautiously and the resulting mixture was stirred vigorously for 30 min. The aqueous solution was extracted with dichloromethane (3 x 200 mL). Combined organic phases were first washed with an equal volume of water, then with 140 mL 10% NaOH, dried over anhydrous Na_2SO_4 , concentrated, and purified by column chromatography (silica gel, eluent: 2% EtOAc: Hexane) to yield 2-chlorobenzoyl ruthenocene as dark yellow crystals. Potassium tertiary butoxide (6.1 g,) was dissolved in 50 mL dimethoxyethane, then, 0.2 mL water was added. To the resultant white slurry, 2-chlorobenzoyl ruthenocene (550 mg) was added. The reaction was refluxed under N_2 for 24 h.

Appendix 2. MATLAB® based data analysis workflow used in MALDI-TOF analysis

**Maldi ToF
Analyze.m**

Appendix 3. Reproducibility of biological properties across different batches

NMR characterization – Representative batch of Dr Yu Du

Dy35-9

Dy36-2

NMR characterization – Representative batch of Dr Zhangyong Si

ZY-B2

ZY-B4

Biological properties

Co-beta-peptide	MIC against SA29213	MTT – 3T3 cells Cell viability (%)	
		100 µg/mL	200 µg/mL
Dy35-9	8 µg/mL	93.2%	79.6%
Dy36-2	8 µg/mL	103.6%	82.4%
ZY-Batch 2	8 µg/mL	94.5%	80.1%
ZY-Batch 4	8 µg/mL	87.3%	76.6%

Appendix 4. Characterization of the clinically isolated MRSA strains.

Designation	HA or CA	CC	Other available characterization
HIP11714	HA	5	USA100 (PFGE) spa repeats TJMGMK; Ridom spa type t062, SCCmec II
HIP11983	HA	5	pulsed-field type USA100; spa repeats TJMBMDMGMK; Ridom spa type t002, SCCmec II
HIP13170	HA	5	USA800; MLST (ST) 5; spa repeats TJMBMDMGMK; Ridom spa type t002, SCCmec IV
HIP13419	HA	5	USA800; MLST (ST) 5; spa repeats TJMBMDMGMK; Ridom spa type t002. SCCmec IV
HIP14300	HA	5	USA100; spa repeats TJMBMDMGMK; Ridom spa type t002, ST5, SCCmec II
HIP15178	HA	5	USA100; spa repeats TJMBMDMGMK; Ridom spa type t002, ST5, SCCmec II
AIS2006032	HA	5	USA100; spa repeats TJMGMK; Ridom spa type t062, SCCmec II
HIP09433	HA	45	MLST sequencing type (ST) 45; eGenomic spa type 15, eGenomic spa repeats A2AKEEMBKB; Ridom spa type t004.
SA MER-S6	HA	5	MLST sequencing type (ST) 5; eGenomic spa type 2, eGenomic spa repeats TJMBMDMGMK; Ridom spa type t002
6820	CA	8	MLST sequencing ST5, Sccmec IV, PVL+
TTSH-478700	HA	22	MLST sequencing ST22
TTSH-671549	HA	22	MLST sequencing ST22
TTSH-478701	HA	22	MLST sequencing ST22
ATCC 700789	HA	5	USA100 (PFGE) SCCmec: Type II, spa type Ridom: t002, spa type Kreiswirth: TJMBMDMGMK
ATCC BAA38	HA	8	USA500, Archaic clone, SCCmec: Type I, spa type Ridom: t051, spa type Kreiswirth: YHFGFMBQBLO
ATCC BAA39	HA	8	ST239-MRSA III, Hungarian clone, Hungarian clone of MRSA, SCCmec: Type III, spa type Ridom: t1053, spa type Kreiswirth: WGKAKAOKAOMQ,
ATCC BAA40	HA	8	ST239 (HDG2), Portuguese clone, SCCmec: Type III, spa type Ridom: t241, spa type Kreiswirth: WGKAOM
ATCC BAA44	HA	8	USA500, ST247-MRSA-1, Iberian clone, SCCmec: Type I, PFGE type: Iberian, spa type Ridom: t051, spa type Kreiswirth: YHFGFMBQBLO