Synthesis of polymeric materials for antibacterial and antibiofilm applications

Li, Jianghua

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**SYNTHESIS OF POLYMERIC MATERIALS FOR ANTIBACTERIAL AND
ANTIBIOFILM APPLICATIONS**

LI JIANGHUA

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2020

**SYNTHESIS OF POLYMERIC MATERIALS FOR ANTIBACTERIAL AND
ANTIBIOFILM APPLICATIONS**

LI JIANGHUA

School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy
2020

Statement of Originality

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Prof. Chan Bee Eng, Mary

Authorship Attribution Statement

This thesis contains material from two papers published in the following peer-reviewed journals where I was the first author.

Chapter 4 is published as Li, J.; Zhang, K.; Ruan, L.; Chin, S. F.; Wickramasinghe, N.; Liu, H.; Ravikumar, V.; Ren, J.; Duan, H.; Yang, L.; Chan-Park, M. B. *Nano Lett.* 2018, 18, (7), 4180-4187. DOI: 10.1021/acs.nanolett.8b01000

The contributions of the co-authors are as follows:

- Prof. Chan Bee Eng, Mary and Dr. Liu Hanbin provided the initial project direction.
- I prepared the manuscript drafts. The manuscript was revised by Prof. Chan Bee Eng, Mary.
- I performed all the laboratory work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- A/Prof. Yang Liang assisted in the analysis of data of biofilm.
- A/Prof. Duan Hongwei assisted in the interpretation of the polymer structure.
- Ms Zhang Kaixi assisted in the preparation and collection of *in vivo* antibiofilm data.
- Ms Ruan Lin assisted in the collection of the MBEC data.
- Ms Chin Seow Fong assisted in the collection of confocal microscopy data.
- Ms Nirmani Wickramasinghe and Ms. Vikashini Ravikumar assisted in the collection of antibacterial data.
- Dr. Ren Jinghua assisted in the collection of *in vivo* toxicity data.

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List of Abbreviations

AST	aspartate transaminase
Ag NPs	silver nanoparticles
AHL	N-acyl L-homoserine lactone
ALT	alanine transaminase
AmpB	amphotericin B
AMPs	antimicrobial peptides
AMPTMA	(3-acrylamidopropyl) trimethylammonium chloride
AST	aspartate transaminase
ATRP	atom transfer radical polymerization
Au NPs	gold nanoparticles
BMA	butyl methacrylate
<i>C. albicans</i>	<i>Candida. albicans</i>
CAT-NPs	catalytic nanoparticles
cCNPs	ciprofloxacin loaded chitosan nanoparticles
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
CM-chitosan	carboxymethyl chitosan
CMC	critical micelle concentration
CTAB	cetyltrimethylammonium bromide
CuBr	copper (I) bromide
CuBr ₂	copper (II) bromide
CV	crystal violet
DA100	dextran- <i>block</i> -poly(AMPTMA)
DA95B5	dextran- <i>block</i> -poly(AMPTMA- <i>co</i> -BMA)
DiSC3(5)	3,3'-dipropylthiadicarbocyanine iodide
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
DPPC	dipalmitoyl phosphatidylcholine

DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
<i>E. coli</i>	<i>Escherichia coli</i>
EPS	extracellular polymeric substances
Fu-cCNPs	fucoidan coated ciprofloxacin loaded chitosan nanoparticles
GPC	gel permeation chromatography
H&E	hematoxylin-eosin
HEK	human embryonic kidney
HFF	human foreskin fibroblasts
HSF	hypertrophic scar-derived fibroblasts
LB	Luria-Bertani
LPS	lipopolysaccharides
MBC	minimum bactericidal concentration
MBEC	minimum biofilm eradication concentration
MDR	multi-drug resistance
MHB	Mueller Hinton Broth
MICs	minimum inhibitory concentrations
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MSPMs	mixed-shell-polymeric micelles
NIR	near-infrared
NMR	nuclear magnetic resonance
NO	nitric oxide
NPs	nanoparticles
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PAA	2-propylacrylic acid
PAE	poly(β -amino ester)
PAGA	poly(2-(acrylamido) glucopyranose)
PAMAM	poly(amidoamine)
PCL	poly(caprolactone)
PDI	polydispersity index
PDMAEMA-C ₄	alkylated poly(2-(dimethylamino) ethy methacrylate)
PEG	poly(ethylene glycol)

PFP	poly{[(9,9-bis(6'-N,N,N-trimethylammonium)hexyl)fluorenylenephenylene]dibromide}
PLGA	poly(DL-lactide-co-glycolic acid)
PMDETA	N,N,N',N',N''-pentamethyldiethylenetriamine
PMPC	poly(2-methacryloyloxyethyl phosphorylcholine)
PSMs	phenol-soluble modulins
PVA	poly(vinyl alcohol)
RAFT	reversible addition-fragmentation chain transfer polymerization
R_g	radius of gyration
R_h	hydrodynamic radius
ROP	ring open polymerization
ROS	reactive oxygen species
SARA	supplemental activator and reducing agent
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SSTIs	skin and soft tissue infections
TA	tannic acid
TBIL	total bilirubin
TEM	transmission electron microscope
THF	tetrahydrofuran
VRE	vancomycin-resistant <i>Enterococcus faecium</i>

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Summary

Antimicrobial resistance has become a global healthcare crisis. Compounded with the evolution of multi-drug resistance, bacteria also develop biofilms to protect themselves, so that biofilm-associated infections are extremely difficult to treat. Antibiotics can be up to 1000-fold less effective to biofilms as compared with the planktonic form. Once developed into the biofilm form, it will become highly resistant to conventional antibiotics. Many antibiotics, natural antimicrobial peptides (AMPs) and synthetic antimicrobial agents have been studied as antibiofilm agents, but the general efficacy is still not high. Further, they usually suffer from the problem of toxicity and limited life span. In this thesis, two series of novel antibiofilm cationic polymeric nanoparticles (NPs) have been developed to show excellent biofilm removal capability.

Firstly, I synthesized polysaccharide-based polymeric NPs made from dextran-*block*-(poly((3-acrylamidopropyl) trimethylammonium chloride (AMPTMA)-*co*-butyl methacrylate (BMA)) (DA95B5). Interestingly, this amphiphilic copolymer DA95B5 self-assembled into NP form which did not have any antibacterial effect but exhibited excellent preformed biofilm removal ability. The antifouling shell of the polysaccharide as well as the NP form, enhanced the biofilm dispersal ability by a mechanism termed “nanoscale bacterial debridement”. Cryo-transmission electron microscope (cryo-TEM) and confocal microscopy showed that these NPs can penetrate into the biofilm and form a coating around the negatively charged bacteria to weaken the cell-biofilm matrix interaction. *In vitro* results showed that the polymeric NPs exhibited antibiofilm ability towards several multi-drug resistant

(MDR) and clinically relevant Gram-positive bacterial strains, with efficacy much higher and/or similar to the conventional standard antibiotics. *In vivo* data corroborated that such NPs possess methicillin-resistant *S. aureus* (MRSA) biofilm removal efficacy that was higher than vancomycin. Further, both *in vitro* and *in vivo* data showed NPs have good biocompatibility with low hemolysis and cytotoxicity. This is the first report of a synthetic intrinsically antibiofilm dispersing agent in contrast to many other such agents which are enzyme-based.

I also presented a novel system (named as FTP NPs) made from biocompatible **F**-127 surfactant, **t**annic acid (TA) and biguanide-based **p**olymetformin (**PMET**), with good antibacterial and antibiofilm activity against MRSA both *in vitro* and *in vivo*. FTP NPs outperformed PMET with around 2-fold more log₁₀ reduction of the MRSA biofilm bacterial cell counts at low concentrations (8-32 µg/mL) *in vitro*, which may due to the antifouling property from the hydrophilic polyethylene glycol (PEG) chain of F-127. Further, in an *in vivo* murine excisional wound model, FTP NPs achieved 1.8 log₁₀ reduction of biofilm-associated MRSA bacteria, which significantly outperform that of vancomycin (0.8 log₁₀ reduction). Moreover, *in vitro* cytotoxicity tests showed FTP NPs has less toxicity than PMET towards mammalian cells; and the *in vivo* data showed that FTP NPs exhibited no acute toxicity to mice with negligible body weight loss and small variation of blood biomarkers at 10 mg/kg via the intravenous injection. These biguanide-based NPs can serve as promising antibiofilm agents against MRSA-associated infections.

Overall, both of these biofilm removal platforms provide exciting opportunities for treatment of multi-drug resistant biofilm infections which may have widespread applications.

Chapter 1 Introduction

1.1 Background

With the discovery of the first antibiotic, penicillin, by Sir Alexander Fleming in 1928, antibiotics have been widely used to terminate bacterial infections.^{1,2} However, the misuse and overuse of antibiotics contribute to the rapid emerging of bacterial antibiotic resistance.³ There are mainly four causes of the bacterial resistance, including (1) reducing the entry of antibiotic by the cell wall, (2) efflux pump, (3) drug inactivation, and (4) modification of the target site of antibiotics.⁴ As a consequence, many multi-drug resistance (MDR) bacteria have emerged and been the threaten to public health.⁵ It is predicted that 10 million people will be killed by these untreatable resistant bacterial infections per year by 2050.⁶ What's worse, bacteria can form biofilm which makes them even much more difficult to be killed.⁷⁻
⁹ Biofilms are estimated to account for up to 80% of bacterial infections and 65% of all nosocomial infections.¹⁰ Biofilms are the main cause of many chronic infections including lung infections in cystic fibrosis, chronic wound infections as well as urinary tract infections of catheters (**Figure 1.1**).¹¹⁻¹⁴

Figure 1.1 Device-related and disease-related biofilm infections. Reproduced with permission from ref. 14. Copyright 2015 Elsevier.

Biofilms are aggregations of microorganisms protected by extracellular polymeric substances (EPS).^{15, 16} The three-dimensional EPS matrix, which mainly consists of exopolysaccharides, proteins and extracellular DNA, can protect the bacteria cells inside biofilms from antibiotics or antimicrobial agents by immobilizing the cell and through enzymatic inactivation of antibiotics or antimicrobial agents.^{17, 18} Further, cells inside biofilms experience lowered metabolic rates so that biofilm bacteria are typically around 1000-fold more resistant to antibiotics than planktonic bacteria.¹⁹ Therefore, new drugs and technologies are urgently needed to eradicate the difficult-to-treat biofilm infections, especially those developed by MDR bacteria.

1.2 Objectives of Thesis

The objectives of this thesis are to develop antibacterial and antibiofilm materials with good *in vitro* and *in vivo* biocompatibility. For the antibacterial materials, cationic polymers have been applied as the killing agents. It is expected that such materials should have good antibacterial ability against MDR bacteria as well as low cytotoxicity towards mammalian cells. For the antibiofilm materials, both synthetic polymeric and supramolecular assembled NPs have been developed to study the antibiofilm ability against MDR bacteria.

Firstly, novel cationic polymers were synthesized based on polysaccharide dextran and cationic monomer AMPTMA. The atom transfer radical polymerization (ATRP) method has been applied to synthesize the first compound dextran-*block*-poly(AMPTMA) (DA100). DA100 showed good antibacterial activity against Gram-positive *S. aureus* including MRSA, but poor biofilm inhibition ability. However, DA100 exhibited biofilm prevention activity against Gram-negative *E. coli* with low antibacterial efficacy. Nevertheless, DA100 incapable to remove preformed biofilm against both Gram-positive and Gram-negative bacteria.

Secondly, in order to remove preformed biofilm, I further modified DA100 to DA95B5 by introducing small amount of hydrophobic monomer BMA. DA95B5 can self-assemble into NPs which don't have any antibacterial effect but excellent preformed biofilm removal ability. The excellent antibiofilm properties might be attributed to the antifouling shell of the polysaccharide as well as the NPs form, which would enhance the biofilm dispersal ability by a mechanism called "nanoscale bacterial debridement". The *in vitro* results showed that the polymeric NPs have the

antibiofilm ability towards several MDR and clinically relevant strains, with the efficacy much higher and/or similar to the conventional standard antibiotics. *In vivo* data also showed that such NPs have MRSA biofilm removal efficacy higher than vancomycin. Further, both the *in vitro* and *in vivo* data showed these NPs have good biocompatibility with low hemolysis and cytotoxicity.

Lastly, supramolecular-based NPs were designed by combining biocompatible **F**-127 surfactant, **TA** and biguanide-based **PMET** (named as FTP NPs). These NPs showed good antibacterial and antibiofilm activity against MRSA by killing bacteria inside and outside biofilm – significantly better than many AMPs or polymers. FTP NPs can remove MRSA USA300 biofilm more effectively than PMET by MBEC assay *in vitro* because of the good biofilm penetration ability of FTP NPs. *In vivo* murine wound infection model also demonstrated that FTP NPs have antibiofilm efficacy much higher compared to antibiotic vancomycin. Moreover, both *in vitro* and *in vivo* data showed NPs have good biocompatibility.

1.3 Organization of Thesis

This thesis has 6 chapters. Chapter 1 is the introduction of research background and objective of this thesis. Chapter 2 is the literature review on antibiofilm agents. Chapter 3 describes the synthesis of cationic polymers as antibacterial agent with biofilm inhibition activity. Chapter 4 is about block copolymeric NPs to remove biofilms of drug-resistant Gram-positive bacteria by nanoscale bacterial debridement. Chapter 5 describes biguanide-derived polymeric NPs for eradicating MRSA biofilm in a murine model. Chapter 6 is the conclusion of the thesis and perspective of future works.

Chapter 2 Literature Review

2.1 Introduction

Biofilm bacteria are 10 to 1000 times more resistant to conventional antibiotics than planktonic state bacteria.^{20, 21} Once a biofilm is formed, its three-dimensional matrix of EPS can protect the biofilm cells from antibacterial agents and immune clearance. A mature biofilm formation can be divided into approximately two stages: the initial bacterial attachment and the three-dimensional biofilm matrix formation stages (**Figure 2.1**).²² Accordingly, many therapeutic strategies have been taken to target the microbial biofilms, such as (1) inhibition of biofilm formation during early stage; and (2) dispersion of mature biofilm by targeting the EPS and/or killing the dormant cells.²² For instances, small molecules, antimicrobial peptides (AMPs), synthetic cationic polymers and NPs have been served as antibiofilm agents.²²⁻²⁴

With the ability to regulate the signal which modulate the biofilm formation and dispersion, small molecules and their derivatives make big contribution to the biofilm treatment.²⁵ AMPs also have been successfully applied into the antibacterial and antibiofilm treatment. Several mechanisms of antibiofilm of AMPs have been illustrated, such as membrane targeting effect, interference with specific biofilm features.²⁶ Synthetic cationic polymers may have antibiofilm activities with similar mechanism of AMPs, but provide broader design of polymers' structures with desired properties.²⁷

Figure 2.1 Opportunities for therapeutic intervention during various stages of the biofilm life cycle. Reproduced with permission from ref. 22. Copyright 2017 Springer Nature.

Nanotechnologies have been widely utilized to combat bacterial infections, including the biofilm associated infections.²⁸ By controlling their sizes, surface properties, shapes and core materials, nanomaterials have become promising antibacterial and antibiofilm agents with mechanisms such as intrinsically bacterial killing as well as delivering antimicrobial drugs (antibiotics, essential oil, nitric oxide, *etc.*).²³ For example, polymeric nanocarriers can serve as drug delivery systems with controllable release of antibacterial cargoes upon interaction with the biofilm matrix through pH changes.^{29, 30} Besides, NPs also can act as antibiofilm agents with photodynamic therapy.^{20, 31} Much more details of current antibiofilm agents have been discussed in the following sections.

2.2 Small Molecules

Small molecules as antibacterial and antibiofilm agents have been well studied.³² For antibiofilm agents, small molecules can inhibit or disperse a biofilm by targeting various essential components in the EPS. For example, quorum sensing plays a significant role in biofilm development and requires signal molecules for activation. For instance, Gram-negative bacteria would use small molecule N-acyl L-homoserine lactone (AHL) to activate quorum sensing and act as transcriptional regulator.³³

D-amino acids are other potent small molecules that can both inhibit biofilm formation and trigger preformed biofilm disassembly.³⁴ TasA fibers can be disengaged to anchor onto bacterial cells by incorporation of a D-amino acid, causing the release of the amyloid fibers that link the cells in the biofilm. In addition, a further study also found that the small molecule norspermidine and its mimics³⁵ can inhibit

biofilm formation and collapse the existing biofilm by directly interacting with the negatively charged EPS (**Figure 2.2**).

Figure 2.2 Norspermidine and norspermidine mimics both inhibit biofilm formation and collapse mature biofilm. Reproduced with permission from ref. 35. Copyright 2013 American Chemical Society.

Furthermore, small molecules have shown antibiofilm ability by binding divalent metal cations and further inhibiting essential protein synthesis. Halogenated phenazines are examples of small molecules that can act as antibiofilm drugs.^{11, 36-40} Bromophenazine (**Figure 2.3**) has been studied to target persistent cells and MDR bacterial biofilms without showing lysis of human red blood cells, indicating good antibiofilm ability and excellent biocompatibility. By measuring the minimum biofilm eradication concentration (MBEC), the halogenated phenazines can eradicate biofilms against MRSA biofilms with an MBEC of 250 μM .³⁷ Moreover, bromophenazine showed biofilm eradication with an efficacy superior to that of the

antibiotic vancomycin. In addition, 2-aminobenzimidazole also shown biofilm inhibition and dispersion ability against Gram-positive bacteria (including MRSA, vancomycin-resistant *Enterococcus faecium* (VRE), and *S. epidermidis*) through a zinc (II)-dependent mechanism.⁴¹

Figure 2.3 Bromophenazine HQ-1 is a small molecule that is active against both planktonic and biofilm MRSA cells via a metal-dependent mechanism. Reproduced from ref. 37 with permission from 2015 The Royal Society of Chemistry.

Although small molecules have shown both biofilm inhibition and dispersion effects, there are still drawbacks to the use of small molecules as therapeutic platforms. Small molecules with antibiofilm abilities that target the EPS have always shown low potency and require combination with other therapies such as

chemotherapy.^{42, 43} Furthermore, metal cations chelating to small molecules have shown narrow-spectrum antibiofilm ability, with the effect mainly on Gram-positive strains.⁴¹ Moreover, *in vivo* studies should also be carried out to prove the efficacy and toxicity of these small molecules.

2.2 Antimicrobial Peptides (AMPs)

AMPs have been widely accepted as a promising weapon to treat MDR bacterial infections.⁴⁴ AMPs usually have amphiphilic structures with positively charged moieties and hydrophobic segments. Furthermore, as shown in **Figure 2.4**,⁴⁵ the antibacterial mechanism of AMPs has mainly been attributed to membrane interactions, including membrane penetration, pore formation, and membrane disruption. With this membrane targeting mechanism, AMPs have shown broad-spectrum antibacterial activity with a reduced chance of resistance development compared to conventional antibiotics.

Figure 2.4 Mechanisms of the antibacterial actions of AMPs. Reproduced with permission from ref. 45. Copyright 2016 Elsevier.

In addition, intracellular activities such as enzyme inhibition, reactive oxygen species (ROS) formation and biomacromolecule inhibition also contribute to the antibacterial effects of AMPs. Nevertheless, many studies have focused on the ability of AMPs to inhibit and/or disperse biofilms.⁴⁶⁻⁴⁹ Although the antibiofilm mechanisms of AMPs are not fully understood, many studies have shown that AMPs can inhibit and disrupt biofilms through mechanisms such as the classical bactericidal effect and/or by targeting biofilm-specific properties (**Figure 2.5**).⁴⁶

Figure 2.5 Possible mechanisms of the antibiofilm activity of AMPs based on the classical bactericidal effects or on the interference with essential attributes of the biofilm lifestyle. Reproduced with permission from ref. 46. Copyright 2015 Elsevier.

For example, Segev-Zarko et al.²⁶ studied biofilm inhibition and degradation using AMPs composed of six lysine residues and nine leucine residues. They showed that the inhibition of the biofilm is due to the reduced bacterial adhesion to surfaces when the AMPs are coated onto the bacteria, and the degradation of preformed biofilm happens by either killing the embedded bacteria or detaching the planktonic live cells.

Specifically, AMPs can act as antibiofilm agents with classical bactericidal effects by targeting bacterial membranes. Recently, short α -helical AMPs have been

synthesized and have shown the ability to eradicate some drug-resistant biofilms.⁵⁰ After optimization by balancing the number of repeat units and hydrophobic amino acids, this short α -helical peptide can penetrate into the membrane of bacteria and further promote biofilm disruption. Another β -Sheet AMP has also been studied for its antibacterial and antibiofilm properties against MDR *P. aeruginosa*.⁵¹ This short AMP, IRIKIRIK (IK8L), showed potent antibacterial activity against Gram-negative bacteria without resistance through a membrane depolarization mechanism. For antibiofilm activity, IK8L showed dose-dependent antibiofilm efficacy. The cell viability of *P. aeruginosa* in the biofilms and the biomass of the biofilms can be reduced to 10% and 35%, respectively, indicating a successful biofilm matrix dispersion effect. Furthermore, RNase 3/ECP peptides,⁵² which combine both antibacterial activity and lipopolysaccharide (LPS) affinity, have high cationicity and amphiphilicity to promote the depolarization of the membrane. These properties contribute to the increased attachment of AMPs onto the EPS of the biofilm and further removal of the preformed biofilm.

Another AMP has shown antibiofilm activity by targeting the different stages of biofilm development. For instance, some AMPs can interfere with the attachment of bacteria to inhibit biofilm formation, and some AMPs can interfere with gene expression to inhibit and/or disperse the biofilm. LL-37 and its derivatives have been well investigated for their ability to inhibit biofilm formation and disrupt mature biofilm.^{10, 53} Some studies^{54, 55} have shown that LL-37 inhibits *P. aeruginosa* biofilm formation by several factors, including (1) reducing *P. aeruginosa* attachment onto the surface, (2) promoting bacterial twitching and surface motility, and (3)

interfering with the quorum-sensing systems (Las and the Rhl systems) of *P. aeruginosa*. Similarly, the small cationic peptide 1037 has been shown to inhibit biofilm formation by reducing the swimming and swarming motilities, promoting twitching and interfering with gene expression during biofilm formation.¹⁰ Moreover, IDR-1018, a synthetic AMP, can disperse a variety of biofilms of Gram-positive and Gram-negative strains.⁵⁶ By targeting and blocking the stress response pathway, IDR-1018 can first disperse the biofilm at low concentrations (0.8 µg/mL) and further kill the dispersed bacterial cells at higher concentrations (10 µg/mL).

Nevertheless, AMPs still suffer challenges and limitations for further clinical applications.⁴⁹ For example, (1) the loss of antibiofilm efficacy when interacting with components of the EPS,^{57, 58} (2) systemic toxicity with low selectivity towards mammalian cells,^{4, 59} (3) enzymatic degradation,^{60, 61} (4) unknown efficacy *in vivo*,⁶² and (5) a high cost of preparation.⁶³ Therefore, much more work is required to improve the bioavailability of AMPs by reducing their toxicity and enhancing their *in vivo* antibacterial and antibiofilm activities.

2.3 Synthetic Cationic Polymers

Cationic polymers have attracted much attention because they can balance the antibacterial effects and biocompatibility that some small molecules or AMPs lack. According to previous studies,⁶⁴⁻⁶⁶ cationic polymers have shown low cytotoxicity to human cells but excellent antimicrobial activity against microbes because they can physically damage the cell wall of the target bacteria, which have distinctly different cell walls from mammalian cells. Examples of such polymers include polysaccharide-based cationic polymers,⁶⁷⁻⁷⁰ polycarbonate,⁷¹⁻⁷⁶ polyacrylate,⁷⁷⁻⁸⁰

polypeptides^{81, 82} and polyamides.^{83, 84} However, most of these studies focused on the killing of planktonic microbes, and few of these polymers have been reported as antibiofilm agents. For example, cationic polymers have shown biofilm inhibition ability by reducing the attachment of bacteria and dispersion activity by killing the microorganism inside the biofilm.⁸⁵⁻⁸⁹ In addition, a few conjugated polymers displayed photodynamic therapy against biofilms by producing ROS with a mechanism different from membrane disruption.

Chitosan and chitosan derivatives have been shown to both inhibit biofilm formation and eradicate mature biofilms. Carboxymethyl chitosan (CM-chitosan) showed biofilm inhibition ability with broad-spectrum antibiofilm activity against both Gram-positive and Gram-negative bacteria as well as fungi.⁸⁸ CM-chitosan inhibited the attachment of bacteria and further prevented biofilm formation. The mechanism of biofilm inhibition may be due to interactions with the bacterial surface and interference with the linkage of bacterial aggregates by neutralizing the surface charge of Gram-positive bacteria *S. aureus* and Gram-negative bacteria *P. aeruginosa*.⁸⁸ In addition, CM-chitosan showed biofilm inhibition against the *Candida* species of fungi by inhibiting the growth of planktonic cells and the attachment of cells.^{90, 91}

In addition to the biofilm prevention effect, some quaternary ammonium-modified chitosan derivatives have also shown enhanced antibacterial activity and enhanced removal of preformed biofilms.⁸⁵ By tuning the ratio of cationic and hydrophobic segments (**Figure 2.6a**), the chitosan derivatives showed different activities against both planktonic bacteria and biofilms of *S. aureus*. Regarding

antibiofilm activity, most of the chitosan derivatives showed a biofilm eradication effect, and a much higher efficacy was found on the derivatives containing much shorter alkyl chains. Furthermore, confocal laser scanning microscopy (CLSM) demonstrated that the antibacterial and antibiofilm activities may be attributed to the membrane disruption mechanism.

Figure 2.6 (a) Antibiofilm activity of chitosan derivatives containing cationic and lipophilic moieties. The *S. aureus* biofilm was stained with SYTO 60 (red) and TOTO 1 (green) to visualize living (red) and dead (green) cells based on membrane integrity. Reproduced with permission from ref. 85. Copyright 2018 American Chemical Society. (b) Nylon-3 polymer inhibits *C. albicans* biofilm formation and kills the mature biofilm. Propidium iodide was used to stain the dead cells (red fluorescence). Reproduced with permission from ref. 94. Copyright 2015 American Chemical Society.

Polyacrylates or polymethacrylates are additional antibiofilm agents that show biofilm elimination ability by targeting the cell membrane.^{86, 92, 93} Using reversible addition-fragmentation chain transfer polymerization (RAFT), cationic amphiphilic methacrylate polymers were synthesized and tested to investigate their antibiofilm ability.⁸⁶ These polymers showed good antimicrobial activity and biofilm eradication efficacy against *S. mutans*. After mature biofilm formation, these polymers were able to reduce biofilm biomass by at least 80% at a concentration of 1000 µg/mL in 2 h. Furthermore, these cationic amphiphilic polymers did not cause significant cytotoxicity to human gingival fibroblasts.

Furthermore, some cationic polycarbonates have been synthesized by ring-opening polymerization (ROP).²⁷ A quaternized polycarbonate was shown to have broad-spectrum antimicrobial activity against clinically relevant Gram-positive *S. epidermidis* and *S. aureus*, Gram-negative *E. coli* and *P. aeruginosa*, and fungus *C. albicans* through a membrane lysis mechanism. More importantly, these polycarbonates can inhibit biofilm growth and disrupt the preformed biofilms of *S. aureus* and *E. coli*. *In vivo* cytotoxicity results showed that the polymers did not cause any significant damage to the important organs of mice at the most effective dose, which makes the polymers an ideal agent for some biofilm-associated infections.

Nylon-3 polymers (poly-βNM) have also shown biofilm inhibition and mature biofilm eradication against amphotericin B (AmpB)-resistant *C. albicans*.⁹⁴ As shown in **Figure 2.6b**, poly-βNM showed antibiofilm ability with efficacy superior to that of AmpB and fluconazole. Further, confocal images indicated that this

cationic poly- β NM showed an antibiofilm effect by targeting the cells in the biofilm, with more dead cells (red) found in the poly- β NM-treated *C. albicans* biofilm. Another synthetic polyamide also showed enhanced antimycobacterial biofilm by the rational design of the hydrophobicity of cationic pendants.⁸⁷ These biocompatible polymers can also kill intracellular mycobacteria without inducing toxicity towards mammalian cells.

Cationic conjugated polymers have also shown good antibacterial and antibiofilm activity because of their photosensitive properties.⁹⁵⁻⁹⁸ The mechanism of bacterial killing and biofilm disruption by these conjugated polymers is mainly attributed to the production of ROS through light irradiation.⁹⁹ For example, as shown in **Figure 2.7**, cationic PFP showed both biofilm inhibition and preformed biofilm dispersion ability.¹⁰⁰ For the inhibition of biofilm formation, cationic PFP can coat the surface of *S. aureus*, resulting in a weakening of the interaction between bacterial cells and preventing biofilm formation. For mature biofilm dispersion, PFP can generate ROS under white light irradiation and kill the bacteria inside the biofilm.

Figure 2.7 Inhibition of biofilm formation and the elimination of mature established biofilm using PFP. Reproduced with permission from ref. 100. Copyright 2017 American Chemical Society.

Overall, although extensive studies of synthetic cationic polymers have been carried out to act as antimicrobial agents, only a few of them have been applied to antibiofilm testing.¹⁰¹ Furthermore, most of these cationic polymers as antibiofilm agents still remain at the stage of *in vitro* evaluation,⁸⁷ and animal models should be studied to show the *in vivo* efficacy as well as less toxicity. Nevertheless, there are a few studies on antibiofilm cationic polymers, but more studies have focused on the antibiofilm abilities of polymeric NPs, especially those made from cationic polymers, providing various opportunities for treating biofilm infections. The details will be fully discussed in the following sections.

2.4 Nanoparticles (NPs)

The emergence of nanotechnology has brought new opportunities for antibacterial and antibiofilm researchers.^{28, 102, 103} Many studies have reported that NPs can serve as good antimicrobial agents.^{23, 104} The advantages of NPs include their small size (usually at 1-100 nm), large surface area, and highly reactive properties.²³ With these unique properties, many NPs have been considered promising antibiofilm agents. NPs have two major actions that can disrupt mature biofilms. On one hand, some NPs have intrinsic antibacterial activity with the ability to penetrate into biofilm and kill the persistent cells by various killing mechanisms; on the other hand, antimicrobial drugs can be entrapped by NPs and then released in a controlled manner to disrupt the biofilm. With the wide variety of methods to synthesize NPs, different materials, including inorganic materials, small molecules, polymers, and biomacromolecules, have been applied to make different NPs to act as antibacterial and antibiofilm agents.²⁸

2.4.1 Metal-Based NPs

Silver (Ag)-based compounds are well-known antibacterial agents that release toxic Ag⁺ ions, which can harm the bacterial membrane and cause DNA damage.^{23, 62} Many studies have focused on Ag NPs as antibiofilm agents.^{28, 105} The size and surface properties of Ag NPs play an important role in antibiofilm activity. On one hand, decreasing the particle sizes can promote the penetrative activity of the Ag NPs.¹⁰⁶ As the particle size decreases, greater attachment of the Ag NPs onto the bacteria can happen, which increases their antibacterial ability. On the other hand, Ag NPs are inclined to self-aggregate when their size is small, which impairs their

antibacterial activity.^{105, 107} Therefore, surface modifications have been applied to further enhance the antibacterial and antibiofilm effectiveness of Ag NPs. For instance, some natural products, including β -cyclodextrin and polyphenol-capped Ag NPs, showed enhanced bacterial inhibition ability without exhibiting normal cell toxicity.^{108, 109} Additionally, functional silver nanocomposites have broad-spectrum antimicrobial activity and biofilm disruption ability (**Figure 2.8**).¹¹⁰ By immobilizing an alkylated poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA-C₄) and/or poly(2-(acrylamido) glucopyranose) (PAGA) onto the Ag NPs, the cationic polymer (PDMAEMA-C₄) can promote the interaction with the negatively charged bacterial membrane and components of the EPS to act synergistically with Ag NPs to enhance the NPs antibacterial and antibiofilm activity against Gram-positive (*S. aureus* and *B. amyloliquefaciens*) and Gram-negative (*P. aeruginosa* and *E. coli*) bacteria. In addition, biocompatible PAGA provides the stability to Ag NPs and further reduces the toxicity of Ag NPs. When combining these three parts, Ag NPs@PAGA/PDMAEMA-C₄ showed excellent mature biofilm disruption both *in vitro* and *in vivo*. Moreover, Ag NPs stabilized by silk fibroin have also shown good antibacterial ability against MRSA, preventing biofilm formation and disrupting the mature biofilm.¹¹¹

Figure 2.8 Functional silver nanocomposites as antimicrobial and biofilm-disrupting agents. Reproduced with permission from ref. 110. Copyright 2017 American Chemical Society.

However, Ag NPs have major concerns that should be further addressed before their clinical application against biofilms. For instance, a major concern of Ag NPs is the unknown toxicity to human cells. In addition, resistance to Ag NPs has emerged in bacteria because of the aggregations of the NPs.¹⁰⁵

Gold NPs (Au NPs) have also been well studied as antibacterial agents through functionalization with different ligands.^{28, 112} Through rational modifications, Au NPs can exhibit broad-spectrum antibacterial activity. For example, a library of cationic Au NPs effectively inhibited MDR strains by varying the hydrophobicity of ligands.¹¹³ These NPs also showed a reduced possibility of inducing resistance due to their membrane disruption mechanism. In addition, antibiotics and/or non-antibiotics capped Au NPs showed antibacterial activity towards MDR bacteria

through binding to disrupting the bacterial membrane.¹¹² Furthermore, another advantage of Au NPs is the ability to generate localized heat by light irradiation for photothermal therapy.¹¹⁴ By combining these killing mechanisms, engineered Au NPs may show good antibiofilm activity towards some clinically important strains. A novel surface-tunable Au NP with a good ability to penetrate biofilms and an enhanced photothermal elimination of the MRSA biofilm was studied.²⁰ Through a simple surface modification with pH-responsive zwitterionic monolayers, Au NPs can promote adherence to bacteria when the acidic environment is formed in the MRSA biofilms. Afterwards, the enhanced accumulation of Au NPs within the MRSA biofilm happens. Using the near-infrared (NIR) light, the accumulated Au NPs can produce heat to ablate the mature biofilm without causing damage to normal tissues. Another strategy to treat biofilms with Au NPs is laser-induced vapor nanobubble formation followed by antibiotic treatment.¹¹⁵ With good penetration of the Au NPs into the biofilm, the NPs can easily penetrate deeply into the biofilm matrix. Once a high-intensity laser is introduced, the Au NPs can produce water vapor nanobubbles and further disrupt the matrix, resulting in an improvement of the antibiofilm efficacy of the antibiotic tobramycin, which is added after laser treatment.

Iron oxide NPs have also shown antibacterial and antibiofilm activity.^{6, 116-118} The main mechanism of iron oxide NPs antibiofilm activity is the production of ROS. In one case (**Figure 2.9**), the polysaccharide dextran was coated onto the surface of iron oxide NPs, which acted as catalytic NPs (CAT-NPs).¹¹⁹⁻¹²¹ With this dextran modification, the CAT-NPs display an improved ability to penetration into the *S. mutans* biofilm matrix. Under the acidic conditions of the biofilm, these CAT-NPs

exhibited peroxidase-like activity to produce ROS, further degrade the EPS and kill bacteria with more than a 5-log reduction in bacterial cell counts compared to the untreated control.

Figure 2.9 CAT-NPs composed of iron oxide NPs coated with dextran result in biofilm disruption via local pH-dependent free radical production, a degraded EPS and bacterial cell death. Reproduced with permission from ref. 121. Copyright 2019 American Chemical Society.

Finally, there are additional metal-based NPs, such as zinc oxide,¹²²⁻¹²⁴ manganese oxide¹²⁵ and copper oxide,¹²⁶⁻¹²⁸ that have shown good antibiofilm ability with mechanisms similar to those mentioned above. However, there are major concerns with these metal-based NPs that should be further addressed before clinical trials. For example, their uncertain toxicity towards host cells and elucidation of their antibacterial and antibiofilm mechanisms.^{102, 129}

2.4.2 Polymeric NPs

There are two main types of polymeric NPs that act as antibiofilm platforms. On one hand, some synthetic and natural polymeric NPs have shown an antibiofilm effect from their innate antibacterial ability. On the other hand, most commonly, polymeric NPs act as nanocarriers to deliver antibacterial drugs into biofilms.

2.4.2.1 Polymeric NPs as Active Antibiofilm Agents

Many natural and synthetic polymeric NPs have exhibited good antibacterial ability and have been widely studied,^{104, 130} but only a few have shown innate antibiofilm activity. For example, chitosan is a natural polysaccharide that has been widely accepted as an antibacterial polymer.^{68, 91, 131, 132} Chitosan NPs prepared by ion gelation with polyanionic sodium triphosphate can reduce *S. mutans* biofilm by killing the bacteria.¹³² Comparing the effects of different molecular weights, low molecular weight chitosan-prepared NPs show higher antibiofilm efficacy than that of the high molecular weight chitosan NPs. Furthermore, synthetic polymeric NPs are another innate antibiofilm agent that are typically made from polymers that can self-assemble into micelles and/or vesicles.²³ Polymeric micelles are composed of amphiphilic polymers that can form core-shell nanostructures. For example, single-chain polymeric NPs made from amphiphilic terpolymers containing poly(ethylene glycol) (PEG) and primary amines and with hydrophobic properties, showed antibiofilm ability with a killing efficacy of >99.99% towards various Gram-negative bacteria including *E. coli* and *P. aeruginosa*.¹³³ Moreover, as shown in **Figure 2.10**, a library of amphiphilic oxanorbornene polymer derivatives showed broad-spectrum antibacterial activity towards MDR strains.¹⁰¹

Figure 2.10 (a) Molecular structures of oxanorbornene polymer derivatives. (b) Polymeric NPs showed antibacterial activity against MDR bacteria and antibiofilm activity without inducing toxicity towards mammalian cells. Reproduced with permission from ref. 101. Copyright 2018 American Chemical Society.

Through rational design of the hydrophobicity that bridges the cationic pendants, these engineered polymers can self-assemble into 10-15 nm NPs in aqueous solution.

Their antibacterial and hemolytic activities are highly dependent on the hydrophobicity of the cationic headgroup. Furthermore, the polymeric NPs showed excellent antibiofilm activity and broad spectrum antibacterial activity against both Gram-positive MRSA and Gram-negative *P. aeruginosa* without inducing toxicity towards mammalian cells.

2.4.2.2 Polymeric NPs as Nanocarriers for Antibiofilm

Although there are only a few reports about polymeric NPs acting as innate antibiofilm agents, and the most common method of dispersion of biofilms by polymeric NPs is their ability to deliver drugs, including antibiotics, antimicrobial agents, essential oils, nitric oxide, etc.^{22, 102} There are many advantages to these delivery systems, including (1) enhancement of the antibiofilm efficacy by protecting drugs from tissue barriers and the biofilm matrix, resulting in increased susceptibility of the bacteria to the drugs. Generally, these delivery systems require good biofilm penetration ability, which is controlled by the size, shape, and surface of the polymeric NPs; (2) entrapment of various drugs, which are usually insoluble in water, unstable under normal conditions, and toxic to human cells by which the release of the drugs to the infected position can be controlled; and (3) smart designs of polymeric NPs that make them suitable to deliver drugs in a targeted manner by adding functional groups.

Antibiotic-Loaded NPs. Biofilms can protect bacterial cells from antibiotics by entrapment in the EPS and/or enzymatic degradation, making the biofilm bacteria even more resistant to antibiotics.^{15, 16} Tremendous efforts have been made to locally deliver antibiotics to the bacteria in biofilms to promote the antibiofilm ability of the

antibiotics.^{22, 134-136} Compared to the free antibiotic, drug-loaded polymeric NPs can protect the antibiotic from clearance and penetrate the tissue barrier and biofilm matrix more efficiently (**Figure 2.11**).¹³⁴ Furthermore, the stability, biocompatibility and controlled release ability of antibiotic-loaded polymeric NPs are promising therapeutic techniques for combating biofilm infections.

Figure 2.11 Mechanisms of antibiotic-loaded polymeric NPs to improve the efficacy of antibiotic drugs for the eradication of bacterial infections via the reduction of self-clearance and inactivation and an increase in penetration through tissue barriers. Reproduced with permission from ref. 134. Copyright 2019 John Wiley and Sons.

First, antibiotic-loaded polymeric NPs can enhance the antibiofilm activity compared to the free drug. Recently, reports have shown that chitosan polymeric NPs are good candidates for preparing nanocarriers to deliver antibiotics into biofilms and further target dormant cells.²³ For example, CM-chitosan prepared NPs

can load vancomycin by physical adsorption. These vancomycin-loaded NPs have good antibacterial activity against vancomycin-resistant *S. aureus* as well as biofilm inhibition ability.¹³⁷ This enhanced antibiofilm mechanism may be due to the ability of chitosan-conjugated vancomycin to penetrate the bacterial cell wall, of which free vancomycin is incapable. The antibacterial and antibiofilm abilities of ciprofloxacin-loaded chitosan NPs (cCNPs) and fucoidan-coated cCNPs (Fu-cCNPs) were also studied.¹³⁸ Enhanced antibacterial activity against *Salmonella* was found by Fu-cCNPs compared to cCNPs and the free drug. Furthermore, both cCNPs and Fu-cCNPs showed similar effects to disperse the *Salmonella* biofilm and showed a higher biofilm dispersion efficacy than that of the free antibiotic.

Second, the controlled release of antibiotics in biofilms is another advantage of polymeric NPs. Biodegradable and biocompatible polymers such as poly(caprolactone) (PCL) and poly(DL-lactide-*co*-glycolic acid) (PLGA) have been widely used as antibiotic delivery systems to treat biofilm infections in a controllable manner.¹³⁵ Levofloxacin-loaded PCL NPs were prepared by a spray-drying technique and applied as a drug delivery system to treat *E. coli* biofilm.¹³⁹ The antibiotic-loaded NPs showed good antibacterial activity as well as biofilm eradication efficacy with 99.9% killing of the bacterial cells in the biofilm. Gentamycin-loaded PLGA NPs were prepared to treat *P. aeruginosa* infections.¹⁴⁰ To promote entrapment efficacy, a solid-in-oil-in-water method was applied to fabricate gentamycin-loaded PLGA NPs that showed a sustainable release of gentamycin for up to 16 days. Gentamycin-loaded PLGA NPs also showed a significant enhancement in biofilm reduction compared to gentamycin alone.

Moreover, these drug-loaded NPs showed reduced gentamycin cytotoxicity at the same dosage required to treat *P. aeruginosa* infections. Amikacin-loaded PLGA NPs also showed good antibiofilm activity against persistent *P. aeruginosa* biofilm without inducing cytotoxicity to mammalian cells.¹⁴¹ Furthermore, to enhance the penetration ability, some hydrophilic polymers, such as chitosan and poly(vinyl alcohol) (PVA), have been used during preparation.¹⁴² With the surface modification of chitosan and PVA, PLGA NPs can enhance the entrapment of colistin and further kill the *P. aeruginosa* preformed biofilm.¹⁴³

Stability as well as a higher drug loading ability of polymeric NPs can also be achieved from nanoplexes of ofloxacin and levofloxacin in complex with hydrophilic dextran sulfate by electrostatic interactions.¹⁴⁴ These NPs showed a high drug loading efficiency of 80% and can be transformed into dry powder for storage for more than one month.

Finally, some smart delivery systems that have been developed not only show the controlled release of antibiotics but also target the components of the biofilm matrix. Enzyme deoxyribonuclease (DNase)-modified ciprofloxacin-loaded PLGA NPs were fabricated by green chemistry to treat *P. aeruginosa* biofilm.¹⁴⁵ These DNase-functionalized NPs can target both the bacteria and the extracellular DNA in the biofilm EPS, providing a promising way to eradicate mature biofilms. Moreover, polymeric NPs made from alginate and chitosan were used as delivery vehicles for tobramycin.¹⁴⁶ Even though a similar antibacterial efficacy was observed for tobramycin and tobramycin-loaded NPs against *P. aeruginosa*, the NPs showed enhanced penetration into the mucus by functionalization with DNase. These novel

antibiotic-loaded NPs provide a strategy to treat *P. aeruginosa* infections in cystic fibrosis with good mucus penetration ability as well as reduced systemic toxicity compared with the free antibiotics.

Another smart polymeric NP is based on stimuli-sensitive groups, such as enzyme and pH-responsive groups. As shown in **Figure 2.12**, hybrid micelles were made from amphiphilic copolymers that loaded a D-amino acid (D-tyrosine) and the antibiotic azithromycin.¹⁴⁷ When interacting with *P. aeruginosa* biofilms, the drugs can be released in a spatiotemporal manner. D-tyrosine can first be released by the pH-sensitive *cis*-aconityl linkers to disperse the dense biofilm matrix; then, the particles decreased in size and became positively charged, leading to an increased biofilm penetration ability; at last stage, azithromycin can be released from the polymers with a lipase-sensitive group, resulting in biofilm disruption.

Figure 2.12 Hybrid micelles disperse biofilms. (i). Release of D-tyrosine from micelles to disperse the biofilms. (ii). Enhancement of the penetration of the micelles into the biofilm matrix and their interaction with the negatively charged bacteria. (iii). In response to bacterial lipases, the grafted

azithromycin is released from the micelles to attack the bacteria and destroy the biofilms. Reproduced with permission from ref. 147. Copyright 2019 Royal Society of Chemistry.

Antimicrobial Agent-Loaded NPs. Some poorly water soluble antimicrobial agents have been loaded into polymeric nanocarriers to improve their antibiofilm ability. Triclosan, one of these antibacterial agents, has been loaded into surface-adaptive polymeric micelles to show enhanced penetration and killing efficacy against *S. aureus* biofilms.¹⁴⁸ These mixed-shell polymeric micelles (MSPMs) can self-assemble into NPs with two shells consisting of hydrophilic PEG and pH-responsive poly(β -amino ester) (PAE). These MSPMs can act as antibiofilm carriers by the following mechanisms: (1) the stealth-like PEG can promote NP penetration deep into the biofilm; (2) PAE can become positively charged under acidic conditions and enhance targeting to the negatively charged bacteria; and (3) the hydrophobic PCL core may be degraded by bacterial lipase and release the trapped triclosan. Overall, these MSPMs are more active than free triclosan or triclosan-loaded PEG-b-PCL NPs (SSPM) against *S. aureus* biofilms.

Furthermore, NPs made from chitosan and zwitterionic poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) have also shown an enhanced ability to penetrate biofilms by the antifouling PMPC.³⁰ Once the NPs diffuse deeper into the matrix, triclosan, which is loaded into the core of chitosan, will be released from the NPs by the decrease in pH. The triclosan-loaded nanocapsules showed enhanced antibiofilm ability compared with triclosan alone against *S. aureus*.

However, one disadvantage of these drug-loaded micelles is that hydrophobic drugs can leak during storage or in the blood circulation. To solve this problem, some systems have been developed, such as the conjugation of hydrophobic drugs onto the backbone of the polymeric micelle by bacterial enzyme-degradable linkages.¹⁴⁹ Due to the preparation of these NPs, the drugs will be only released upon meeting the bacteria, allowing for longer storage and the controlled release at the infected site. One example is triclosan linked to PEG-*b*-PAE micelles by biodegradable ester linkages. These drug-conjugated micelles showed good biofilm penetration effects because of the antifouling PEG and pH-sensitive PAE. By administering these PEG-PAE-triclosan micelles, the ester-linkages can be degraded, and triclosan will be released to kill a broad-spectrum of biofilms including MRSA, *S. mutans* and *E. coli*. Moreover, the results also demonstrated that these drug conjugated PEG-PAE-Triclosan micelles showed better antibiofilm effects than free triclosan and the conventional triclosan-loaded micelles at equal loading concentrations.

Essential Oil-Loaded NPs. Essential oils are hydrophobic chemicals extracted from plants that have been widely used as antibacterial and antifungal agents in the cosmetic, laundry and food industries.^{150, 151} The antimicrobial activity of essential oils may be due to their ability to (1) disrupt the permeability of the membrane, (2) interact with proteins in the membrane, (3) affect respiratory processes and (4) inhibit the synthesis of DNA and proteins.¹⁵² Moreover, many essential oils have been used as antibiofilm agents against various microorganisms.¹⁵³ For instance, *Laurus nobilis* L. essential oil showed good biofilm prevention ability against oral *S. aureus* at a concentration that was below the MIC.¹⁵⁴ Furthermore, the same essential

oil showed good antifungal activity, biofilm inhibition activity and mature biofilm reduction activity against *Candida* spp.¹⁵⁵ However, the natural hydrophobicity, poor stability and volatility of essential oils limited their applications to treat biofilm infections.¹⁵⁶ To enhance their bioavailability, polymeric NPs have been used to encapsulate essential oils to combat biofilms of various microorganisms.

PLGA- and phosphatidylcholine-prepared NPs were used to encapsulate the essential oil carvacrol by the solvent displacement method.¹⁵⁷ These carvacrol-loaded NPs can diffuse through mucus layers and further alter the properties of *S. epidermidis* biofilms. As a consequence, these NPs can penetrate deep into the biofilm and release the antimicrobial agent to disrupt the preformed biofilm. To improve the stability and antibiofilm effects of these NPs, crosslinked nanocomposites (**Figure 2.13a**) were prepared from carvacrol, poly(maleic anhydride-*alt*-octadecene) (p-MA-*alt*-OD) and poly(oxanorborneneimide) bearing a tetraethylene glycol monomethyl ester, guanidine and amine (PONI-GAT) using the essential oil-in-water method.¹⁵⁸ These NPs showed an enhanced ability to penetrate biofilms and good stability during storage as well as superb biocompatibility. Furthermore, the fast release of carvacrol in 3 h caused the NPs to eradicate multiple biofilms from Gram-positive MRSA and Gram-negative *E. coli* and *P. aeruginosa*.

Figure 2.13 (a) The carvacrol oil-in-water crosslinked polymeric nanocomposite penetrates and eradicates the MDR biofilm. Reproduced with permission from ref. 158. Copyright 2017 American Chemical Society. (b) pH-activated polymeric NPs for the controlled topical delivery of farnesol to disrupt *S. mutans* biofilm. Reproduced with permission from ref. 29. Copyright 2015 American Chemical Society.

However, these cross-linked nanocomposites are nonbiodegradable and could possibly remain in the body to cause unwanted side effects. Therefore, a biodegradable crosslinked nanocomposite was prepared by entrapping carvacrol oil

using the same oil-in-water emulsion method.¹⁵⁹ These nanocomposites provided multiple degradable linkages, including ester bonds, dithiol linkers, and thiol-maleimide linkages. The antibacterial activity was determined and showed broad-spectrum killing effects. Furthermore, these nanocomposites showed good antibiofilm effects against both Gram-positive and Gram-negative biofilms in a coculture model. The results also showed a good biofilm penetration effect from these nanocomposites and a significant reduction (4-log reduction) of bacterial counts in the biofilm without causing an obvious change in mammalian cell viability. Overall, both the nondegradable and biodegradable nanocomposites showed good antibiofilm ability to be promising therapeutic agents for wound infections.

Moreover, smart polymeric NPs were studied to deliver essential oils with a stimuli-triggered releasing effect. For example, as shown in **Figure 2.13b**, pH-adaptive polymeric NPs consisting of 2-(dimethylamino)ethyl methacrylate (DMAEMA), butyl methacrylate (BMA), and 2-propylacrylic acid (PAA) (p(DMAEMA)-*b*-p(DMAEMA-*co*-BMA-*co*-PAA)) were synthesized to encapsulate farnesol to eradicate oral biofilms.²⁹ In this smart system, amphiphilic copolymers can first self-assemble into cationic NPs with p(DMAEMA) as the shell and p(DMAEMA-*co*-BMA-*co*-PAA) as the hydrophobic core. With the loading of the hydrophobic farnesol into the hydrophobic core of the copolymer, these drug-loaded cationic NPs can interact with the negatively charged components in the biofilm EPS. Furthermore, the release of farnesol is mainly controlled by the pH gradient in the biofilm. Once the acidic conditions are met, the pH-responsive PAA and p(DMAEMA) will change from being hydrophobic to hydrophilic and release

the hydrophobic essential oil. The results showed that the farnesol-loaded polymeric NPs can disrupt *S. mutans* biofilms with an efficacy that is 4-fold greater than that of free farnesol.

Nitric oxide (NO)-Loaded NPs. NO, a promising biofilm dispersion molecule, has also been widely studied.¹⁶⁰⁻¹⁶⁴ Molecular analysis revealed that NO can induce biofilm dispersion by regulating the level of the intercellular concentrations of cyclic di-GMP.²² However, gaseous NO suffers from the problems of a short half-life, limited storage, and quick release. To overcome these challenges, polymeric NPs can load NO donors and release NO to prevent and disperse the biofilms of many microorganisms.^{165, 166}

Star polymer-based NPs were first synthesized by RAFT polymerization (**Figure 2.14**).¹⁶¹ Gaseous NO then reacted with the secondary amine in the polymeric NP to form N-diazeniumdiolate groups located in the core of the NPs. When testing the biofilm inhibition ability towards *P. aeruginosa*, NO star polymers showed 90% and 95% biofilm biomass reduction at concentrations of 100 and 400 µg/mL, respectively. Further, confocal microscopy was used to examine the biofilm dispersion ability of NO star polymers and it was demonstrated that these NPs can disperse the preformed biofilms of *P. aeruginosa*.

Figure 2.14 Synthesis of P(OEGA)-b-P(VDM) core cross-linked star polymers followed by spermine and NO donor conjugation. Reproduced with permission from ref. 161. Copyright 2014 American Chemical Society.

Furthermore, poly(amidoamine) (PAMAM) dendrimers modified with propyl, butyl, hexyl, octyl and dodecyl alkyl chains can react with NO to form N-diazeniumdiolate NO donors.^{160, 164} When assessing the antibiofilm activity of NO-loaded PAMAM dendrimers, several conclusions have been drawn, including (1) longer alkyl chain-modified dendrimers show enhanced antibiofilm ability, which may be due to the greater interaction with the cell membrane and promotion of biofilm penetration; (2) released NO can further enhance antibiofilm efficacy for alkyl-modified PAMAM dendrimers that are not capable of penetrating into the biofilm; and (3) these NO-releasing dendrimers have shown broad-spectrum

antibiofilm activity against both Gram-negative *P. aeruginosa* and Gram-positive *S. aureus* and *S. mutans*.

2.4.3 Lipid NPs

Liposomes have been recognized as promising candidates to eliminate biofilm infections due to their various advantages, including (1) enhanced antibacterial activity by fusing with bacterial membranes to further deliver drugs, (2) protection of the antimicrobial agents from the biofilms, (3) coencapsulation of different antimicrobial agents, and (4) enhanced penetration effects.^{22, 102, 135}

Fusogenic liposomes have the ability to fuse with bacterial phospholipid membranes and show enhanced killing activity against both gram-negative and gram-positive bacterial biofilms.²³ Daptomycin-loaded nanoliposomes have been evaluated to test the antibiofilm ability towards *S. aureus*.¹⁶⁷ Made from soy phosphatidylcholine and sodium cholate, these nanoliposomes showed rapid and excellent antibacterial activity against *S. aureus* by fusion of the liposomal lipid bilayer and bacterial membrane. Furthermore, these nanoliposomes showed good skin permeability and inhibited biofilm formation by the enhanced penetration of daptomycin.

In addition, liposomes can protect the drugs inside the NPs from interacting with the biofilm components.¹⁶⁸⁻¹⁷⁰ Therefore, amikacin-loaded liposomes, which are made of dipalmitoyl phosphatidylcholine (DPPC) and cholesterol, have been used to deliver amikacin to treat *P. aeruginosa* lung infections. These liposomes showed a good ability to penetrate biofilms and release amikacin at the infected site. *In vivo*

data also demonstrated a significant enhancement in the antibiofilm ability compared to the free drug.

With the vesicle structure, liposomes can entrap hydrophilic drugs in their aqueous cores and hydrophobic drugs in the lipid bilayer.¹⁷¹ 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol-containing liposomes can entrap both the hydrophilic antibiotic tobramycin and the lipophilic metal bismuth.^{172, 173} As a result, the tobramycin and bismuth contained within the liposome showed good biofilm penetration ability and killed the Gram-negative bacteria *P. aeruginosa* inside biofilms with an efficacy that was much higher than that of the free tobramycin and bismuth. Furthermore, these drug-loaded liposomes also showed reduced toxicity against lung cells compared to the free drugs.

Some surface-charged liposomes have shown different antibiofilm effects.^{23, 102, 174} For example, three different surface charged liposomes were used to entrap clarithromycin.¹⁷⁵ The positively charged liposomes consisted of DPPC, didecyldimethylammonium bromide (DDAB), and cholesterol; the negatively charged liposomes were made of DPPC, dicetyl phosphate (DCP) and cholesterol; and the uncharged liposomes were composed of DPPC and cholesterol. All these clarithromycin-loaded liposomes showed enhanced antibacterial activity compared to the free drugs against *P. aeruginosa*. However, the positively charged liposomes showed the highest antibacterial efficacy, which may be due to electrostatic interactions and enhanced fusion between the liposomes and the bacterial membrane. Furthermore, these positively charged liposomes also showed better antibiofilm activity than the other formulations by completely eradicating the biofilms. This

outstanding performance may be attributed to the interaction of the oppositely charged liposomes and bacterial membrane, resulting in the enhanced biofilm penetration effect and the release of the antibiotics into the biofilm.

Overall, liposomes provide promising antibiofilm activity with various advantages, including multiple drug encapsulation, enhanced antibiofilm activity compared to the free drugs and reduced toxicity. However, only a few have been tested for their *in vivo* antibiofilm efficacy and biocompatibility.

2.5 Conclusions

There is an urgent to develop novel strategies to treat biofilm-associated infections. In this chapter, I highlighted the latest development of antibiofilm agents, such as small molecules, AMPs, synthetic cationic polymers and NPs systems.²²⁻²⁴ Small molecules can act as signals to regulate the biofilm formation and/or dispersal; or chelate metal ions and further inhibit protein synthesis.²⁵ AMPs and synthetic polymers act as antibiofilm agents with several mechanisms including membrane targeting effect, interference with specific biofilm features.^{26 27} Even though, small molecules, AMPs, and synthetic cationic polymers may suffer some problems.¹⁶ As shown in **Figure 2.15**, biofilm matrix may inactive antibacterial agents by trapping them and/or enzymatic degradation. Besides, the poor penetration ability of these compounds also prevents them to kill the bacteria imbedded in deeper biofilm matrix, resulting in limited antibiofilm efficacy.²³

Figure 2.15 (a) Intrinsic antimicrobial-resistance and (b) poor penetration of antimicrobials into biofilms form the two main reasons for the recalcitrance of infectious biofilms to antimicrobial treatment. Reproduced with permission from ref. 23. Copyright 2019 Royal Society of Chemistry.

In the effort to develop next-generation antibiofilm agents to meet both efficacy and biocompatibility requirements, nanotechnology has been attracted much attention.^{23, 130} The size, surface morphology and charge of NPs can be tuned to enhance penetration into the biofilm matrix.²³ Many metal-based nanocomposites such as Ag NPs, Au NPs and iron oxide NPs have showed antibacterial and antibiofilm activity with mechanisms including (1) toxic ion releasing, (2) membrane

disruption, and (3) ROS production.^{28, 62} However, some major concerns of these metal-based NPs still remained. For examples, the uncertain toxicity towards the host cells.

Further, polymeric NPs also showed good antibacterial ability as well as enhanced antibiofilm activity.¹⁰² Except for the intrinsically antibacterial and antibiofilm polymeric NPs, many polymeric NPs can serve as nanocarriers to deliver drugs (such as antibiotics, essential oils, NO, *ect.*). By sophisticated design of these NPs, they can deliver different drugs on demand by different triggers.²² Overall, nanomaterials provide a promising alternative as antibacterial and antibiofilm agents with the intrinsically therapeutic activity as well as drug delivery ability. However, the safety of NPs still remains as a concern and also lacks *in vivo* efficacy tests, which hamper nanomaterials applications in clinical trials. Therefore, the developments of nanomaterials with good antibacterial and antibiofilm abilities as well as excellent biocompatibility become the first priority recently.

Chapter 3 Synthesis of Antibacterial and Biofilm Prevention Cationic Polymer with Biocompatibility

3.1 Introduction

Since the discovery of antibacterial agent, more than billions of lives were saved from serious infection of pathogens. However, the microbes have been developed strong resistance to drugs.³ Cationic polymers might be promising alternative antimicrobial agents against these drug-resistant microbes because of their ability to physically damage the cell wall mechanisms quite different from the traditional antibiotics.¹⁷⁶ In the past two decades, enormous effort was donated into the development of AMPs,¹⁷⁷⁻¹⁸² but always suffered problems such as high cytotoxicity, low stability to protease as well as high costs for producing.¹⁸³ Comparing to peptides, cationic polymers take the advantages of low cost for manufacturing and easily modification with lots of different chemical methods.

In this chapter, I studied the synthesis and application of cationic polymer dextran-*block*-poly(AMPTMA) in antibacterial and biofilm inhibition activity. Dextran is a polysaccharide with good biocompatibility and low cost.¹¹ Furthermore, dextran is easy to be modified at the end group to make a block copolymer with various methods, including ATRP, RAFT polymerization, and ROP as well as directly coupling with amine group.¹¹ Herein, ATRP was utilized to synthesize a series of dextran-*block*-poly(AMPTMA). The optimized block copolymer DA100 can target the microbial cell wall of Gram-positive bacteria *S. aureus* and then show its cationic moieties to disturb the cytoplasmic membrane of bacteria. Furthermore, DA100 is a kind of low efficiency bacterial killing but good biofilm inhibition agent against the

Gram-negative bacteria *E. coli K12*. The low killing effect is due to the weakly binding of the polymer to the surface of *E. coli K12*, whereas the excellent biofilm inhibition property is mainly due to the antifouling effect of dextran.^{19,20,21}

3.2 Experimental Section

Materials

Dextran ($M_n=6000 \text{ g mol}^{-1}$), di-tert-butyl dicarbonate (BOC_2O , 98%), ethylene diamine (99%), 2-bromoisobutyrylbromide (98%), (3-acrylamidopropyl)trimethylammonium chloride solution 75 wt. % in H_2O , sodium cyano-borohydride (NaCNBH_3 , 98%), copper (I) bromide (CuBr , 99%), copper (II) bromide (CuBr_2 , 99%), Ethyl α -bromoisobutyrate (EBiB , 98%), N,N,N',N'',N'' -pentamethyldiethylenetriamine (PMDETA, 99%), dimethyl sulfoxide (DMSO), 3,3'-dipropylthiadicarbocyanine iodide: DiSC3(5), and tetrahydrofuran (THF) were obtained from Sigma-Aldrich; it was stirred overnight with CaH_2 and distilled prior to use.

Supplemental Activator and Reducing Agent (SARA) ATRP of Poly(AMPTMA)

SARA ATRP of poly(AMPTMA) followed this procedure: 33 mg EBiB (0.017 mmol) was dissolved in 2 mL DMSO followed by adding AMPTMA solution (75 wt. % in water, 1.7 mmol, 100 equiv.), 2.4 mg CuBr (0.017 mmol, 1.0 equiv.), 1.9 mg CuBr_2 (0.0085 mmol 0.5 equiv.), and 14.7 mg PMDETA (0.0085 mmol, 0.5 equiv.). Next, Cu (0) wire was added and then deoxygenated by purging with argon. Then the mixture was reacted at 50 °C in oil bath for 24 h. Then dialyzed against DI water with 6-8 kDa cutoff dialysis membrane. After dialysis the product was obtained after lyophilization.

Synthesis of Dextran-Br Macroinitiator

The macroinitiator dextran-br was synthesized according to a reported procedure.¹⁸⁴ Dextran-br was synthesized by reductive amination between terminal anomeric aldehyde of dextran with a terminal ω -amino-isobutyrylbromide in DMSO/H₂O promoted by NaCNBH₃/Et₃N. The crude product was dialyzed against deionized water using Mw= 3500 Da cutoff tube for 5 days. After lyophilization, the ¹H NMR is in agreement the published data.

SARA ATRP of Dextran-*block*-Poly(AMPTMA) Copolymers

Take DA100 as an example: in a Schlenk tube, 0.1 g dextran-br (0.017 mmol) was dissolved in 3mL DMSO followed by adding AMPTMA solution (75 wt. % in water, 1.7 mmol, 100 equiv.), 2.4 mg CuBr (0.017 mmol, 1.0 equiv.), 1.9 mg CuBr₂ (0.0085 mmol 0.5 equiv.), and 14.7 mg PMDETA (0.0085 mmol, 0.5 equiv.). Next, Cu (0) wire was added and then deoxygenated by purging with argon. Then the mixture was reacted at 50 °C in oil bath for 24 h. Then dialyzed against DI water with 6-8 kDa cutoff dialysis membrane. After dialysis the product was obtained after lyophilization.

Characterization

The characterization of synthesized compounds was measured by ¹H NMR spectra (Bruker Avance II 300MHz NMR Spectrometer). Gel permeation chromatography (GPC) equipped with a refractive index detector to measure the molecular weights and polydispersity. The prepared samples (1 mg/mL) were injected into Water's GPC system equipped with two ultrahydrogel columns using acetate buffer (pH ~4.5)

as the elute. Narrow distributed pullulan standards were used for the calibration curve.

Zeta Potential Measurements

The assay was performed as described previously with minor modification.¹⁸⁵⁻¹⁸⁷ Mid-log phase bacteria in PBS were incubated with DA100 for 20 min at 37 °C (the polymer concentrations range from 0 to 500 µg/mL). After incubation, the unbound polymer was removed by centrifugation (10000 rpm, 2min). The obtained pellets were washed once and re-suspended with 1 mL PBS and the suspensions were kept on ice for zeta potential measurements (Malvern Instruments, Malvern, U.K.). As negative controls, untreated bacteria were also incubated under exactly the same conditions.

Cytoplasmic Membrane Depolarization

Bacterial cells (10^{8-9} CFU/mL) were centrifuged and washed using 5 mM HEPES buffer (pH 7.8). Then bacteria were prepared to reach a concentration of 10^7 CFU/mL. For Gram negative cells *E. coli*, bacteria were treated with 0.2 mM EDTA before adding DiSC3(5). DiSC3(5) solution was added to 2 mL bacteria suspension in a 1 cm cuvette to achieve a final concentration of 100 nM. DiSC3(5) dye was gradually quenched at room temperature for 30 minutes. The quenching time for different bacteria may be different and was adjusted accordingly. Polymer solution (100 µg/mL) were added under stirring. Fluorescence intensity were measured by spectrometer at $\lambda_{ex}=622$ nm and $\lambda_{em}=670$ nm. Sampling interval was set at 1.5 seconds.

Minimum Inhibitory Concentrations (MICs)

Bacteria cells were grown and diluted in Mueller Hinton Broth (MHB) to 10^5 - 10^6 CFU/mL. A stock polymer solution (10.24 mg/mL) was prepared and diluted in MHB with two-fold serial dilution in a 96-well microplate. After that, 50 μ L bacterial suspension in MHB (10^5 - 10^6 CFU/mL) was added into the polymer solution and the total volume in each well was 100 μ L. After incubated at 37 °C for 16-18 h, the OD was recorded at 600 nm wavelength using a TECAN microplate reader. Bacteria without polymer solution was positive control and MHB was the negative control.

Hemolysis Assay

Human red blood cells were collected and prepared in tris buffer (pH 7.2) to a concentration of 5 % in volume ratio. Polymer solution in tris buffer with two-fold dilutions was prepared and added into 50 μ L blood cells solution to reach a total volume of 100 μ L. After incubated at 37 °C for 1 h, the 96-well microplate was centrifuged for 10 min at 1,000 $\times$ g. 80 μ L of the supernatant were taken out and added into 80 μ L of tris buffer to achieve total volume of 160 μ L. Hemolytic activity was determined at OD=540 nm using a TECAN microplate reader. The positive control is 0.1 % Triton X-100 and the negative control was tris buffer. The hemolysis percentage was calculated based on following equation:

$$\% \text{ Hemolysis} = \left(\frac{O_p - O_b}{O_t - O_b} \right) \times 100$$

where O_p is the OD value of polymer treated solution, O_b is OD value of negative control, and O_t is the OD value of positive control.

***In Vitro* Biocompatibility Studies**

3T3 mammalian cells were seeded in a 96-well microplate (1×10^4 cells/well) and cultured at 37 °C for 24 hours. A stock polymer solution (10.24 mg/mL) was

prepared and diluted in DMEM complete medium with two-fold serial dilution. After that, 200 μ L of polymer solution at desired concentrations was added to the cells in the 96-well plate. After incubated at 37 °C for 24 hours, the polymer-containing DMEM solution was discarded and the cells were washed with PBS. After that, 100 μ L 1 mg/mL MTT solution in DMEM were added into the 96-well plates and incubated for another 4 h. Finally, the MTT medium was discarded followed by addition of DMSO (100 μ L). The cell viability was determined by measuring OD at 570 nm wavelength.

Analysis of Biofilm Inhibition

The assay was performed as described previously with minor modification¹⁶. Briefly, all the tested bacterial strains were cultured in TSB medium and diluted to 10^6 - 10^7 CFU/mL. The polymer solutions were prepared in the same medium with two-fold serial dilution and added 75 μ L of the polymer solution into a 96-well microplate. After that, 75 μ L of bacterial dilution were added into per well in the 96-well microplate to achieve a final volume of 150 μ L. The microtiter plate was incubated for 24 hours at 37°C. After incubation, the bacterial suspension was discarded and the microplate was rinsed DI water twice followed by adding 125 μ L of 0.1 % crystal violet (CV). After that, the microplate was kept at room temperature for 10 min followed by washing DI water twice. A 125 μ L of 30 % acetic acid solution was added into 96-well plates and kept for 15 min to dissolve CV. Lastly, a TECAN microplate reader was used to measure OD₅₅₀ value to determine the biomass.

3.3 Results and Discussion

The reactant ω -amino-isobutyrylbromide was synthesized from ethylene diamine in 3 steps (**Scheme 3.1**). The only one amino group was firstly protected with (tert-butyloxy) carbonate (Boc) and followed by another amino group was acylated with 2-bromoisobutyrylbromide to achieve unsymmetrical protected ethylene diamine. The required ω -amino-isobutyrylbromide was obtained after deprotection of Boc group under acidic reaction condition. The dextran macro-initiator was synthesized by oxime click reaction amination of the anomeric end of with the bromoisobutyryl group at 1.89 ppm (peak k, **Figure 3.1**).

Scheme 3.1 Synthesis of dextran-*block*-poly(AMPTMA) by SARA ATRP.

Figure 3.1 ^1H NMR spectra of (a) dextran and (b) macro-initiator in DMSO-d_6

The ATRP was then conducted to synthesize a series of dextran-*block*-poly(AMPTMA) (DA20, DA50, DA100, DA150, and DA200) by using the $\text{Cu}(0)$ - CuBr - CuBr_2 -PMDETA catalyst complex. Take DA100 as an example, ^1H NMR

spectra (**Figure 3.2**) showed that the anomeric proton (4.95 ppm) and the protons of the glucosidic unit (3.4-4.0 ppm) are from the dextran ($M_n = 5700$ Da) backbone, and the rest peaks are protons from poly(AMPTMA). The degree of polymerization ($DP_n=64$) of the AMPTMA in the diblock copolymer can be calculated from the ratio of the total areas of peaks ($e+g+h$) to the areas of the protons of glucosidic unit (3.4-4.0 ppm).

Figure 3.2 ^1H NMR spectrum of DA100 in D_2O

Furthermore, these dextran-*block*-poly(AMPTMA) copolymers were characterized by GPC and the data (**Figure 3.3** and **Table 3.1**) showed clearly that the molecular weight shift to higher region with the increasing feeding ratio of AMPTMA. GPC traces were also symmetrical with no residual macroinitiator.

Overall, ^1H NMR and GPC results demonstrated the successful synthesis of dextran-*block*-poly(AMPTMA) copolymers.

Figure 3.3 GPC traces for dextran-*block*-poly(AMPTMA) copolymers.

Table 3.1 GPC data for dextran-*block*-AMPTMA copolymers

Polymers	M_n (kDa)	M_w (kDa)	PDI
DA20	6.40	9.37	1.46
DA50	8.75	14.80	1.69
DA100	15.47	24.28	1.57
DA150	16.34	29.90	1.83
DA200	18.04	33.74	1.87

The effect of the cationic chain length of these diblock copolymers on antibacterial activity and biocompatibility was first studied. As shown in **Table 3.2**, the MICs values of these copolymers decrease when the cationic chain increases. For Gram-positive bacteria (*S. aureus* ATCC29213 and MRSA BAA40), MICs values of copolymers decrease from 512 to 8-16 $\mu\text{g/mL}$ when the feeding amount of AMPTMA is above 100. However, as for Gram-negative bacteria PAO1, the MICs value of DA200, which has the highest feeding amount of AMPTMA, still remains as high as 256 $\mu\text{g/mL}$; and as for Gram-negative bacteria *E. coli* ATCC8739, the MICs values of copolymers decrease gradually from 512 to 32 $\mu\text{g/mL}$ when the feeding amount of AMPTMA changes from 20 to 200.

Table 3.2 Minimum inhibitory concentrations (MICs: $\mu\text{g/mL}$) of dextran-*block*-poly(AMPTMA) copolymers series against bacterial strains.

Sample names	MICs ($\mu\text{g/mL}$)			
	<i>E.coli</i> (ATCC 8739)	<i>S.aureus</i> (ATCC 29213)	<i>S.aureus</i> (MRSA BAA40)	<i>P. aeruginosa</i> PAO1
DA20	>512	>512	>512	>512
DA50	>512	64	32	>512
DA100	128	16	8	256
DA150	64	16	8	256
DA200	32	16	8	256

The cytotoxicity of these dextran-*block*-poly(AMPTMA) copolymers was further tested against mouse embryonic fibroblast 3T3 cells. The results (**Table 3.3**) show that the biocompatibility of these diblock copolymers against 3T3 cells is correlated to the cationic AMPTMA. With the increasing amount of cationic AMPTMA, the cytotoxicity of these copolymers is also enhanced. Among them, DA100 shows the best antibacterial activity as mentioned above while keeping biocompatibility with the with 70% inhibitory concentration (IC_{70}) $>100 \mu\text{g/mL}$ against 3T3 cells. Therefore, DA100 copolymer was selected as optimized compound for further study.

Table 3.3 Cytotoxicity of dextran-*block*-poly(AMPTMA) copolymers against 3T3 cells.

Sample names	Cell viability (%)	
	100 $\mu\text{g/mL}$	500 $\mu\text{g/mL}$
DA20	79.7 ± 14.0	71.5 ± 0.2
DA50	83.6 ± 0.1	63.8 ± 0.1
DA100	70.4 ± 0.1	43.0 ± 0.1
DA150	56.4 ± 0.0	38.4 ± 0.0
DA200	55.5 ± 0.0	36.6 ± 0.1

To compare the antibacterial activity of counterpart of DA100, homocationic polymer A100 was synthesized by the same SARA ATRP method. ^1H NMR (**Figure 3.4**) and GPC data (**Figure 3.5**) show that the degree of polymerization of A100

($M_n=9,600$, $DP_n=60$) was closed to that of the AMPTMA in DA100 ($DP_n=64$), indicating the comparable amount of the cationic moiety of these two polymers.

Figure 3.4 ^1H NMR spectrum of A100 in D_2O

Figure 3.5 GPC traces for (a) A100 and (b) DA100 with calibration plots.

Furthermore, A100 and DA100 show similar antimicrobial activity (MICs: 8-16 $\mu\text{g/mL}$) against Gram-positive bacteria (*S. aureus*, including MRSA BAA40), but moderate efficacy (MICs: 128-256 $\mu\text{g/mL}$) against the Gram-negative species *E. coli* (**Table 3.4**). Further, as shown in **Table 3.4**, both A100 and DA100 are non-hemolytic, with concentrations for 10% hemolysis of human red blood cells (HC_{10}) > 20,000 $\mu\text{g/mL}$. *In vitro* acute toxicity testing show that the 50% inhibitory concentrations (IC_{50}) of DA100 towards 3T3 mammalian cells is $141.7 \pm 17.4 \mu\text{g/mL}$, which is much higher than that of A100 ($65.2 \pm 13.2 \mu\text{g/mL}$). It is believed that the addition of the polysaccharide dextran can slightly reduce the cytotoxicity of the copolymers. Therefore, the newly synthesized DA100 shows potent antibacterial activity against Gram-positive *S. aureus* including MRSA as well as low cytotoxicity and non-haemolytic.

Table 3.4 Biological properties of A100 and DA100: MICs and hemolytic concentration for 10% red blood cell lysis (HC_{10} , $\mu\text{g/mL}$) and the 50% inhibitory concentration with 3T3 cells (IC_{50} , $\mu\text{g/mL}$).

Samples	MIC: $\mu\text{g/mL}$				HC_{10} ($\mu\text{g/mL}$) RBC	IC_{50} ($\mu\text{g/mL}$) 3T3
	<i>S. aureus</i> (MRSA BAA-40)	<i>S. aureus</i> (ATCC 29213)	<i>E. coli</i> (ATCC 8739)	<i>E. coli</i> K12		
A100	8-16	16	128	256	>20000	65.2 ± 13.2
DA100	8-16	16	128	256	>20000	141.7 ± 17.4

Further, in order to understand the killing mechanism of DA100 with planktonic Gram-negative *E. coli* and Gram-positive *S. aureus*, I first titrated the bacteria with increasing concentrations of DA100 and measured the zeta potential of the bacteria. The zeta potential provides direct evidence of the binding of cationic polymers to the surface of bacteria (**Figure 3.6**). With the addition of DA100, the zeta potentials of *E. coli* became more positive, changing from -15 mV to +5 mV indicating that DA100 aggregates on *E. coli* surface. However, with similar DA100 addition, the zeta potential of MRSA BAA40 remained negative without distinctly change. We hypothesized that the Gram-positive *S. aureus* has a comparatively thick and porous peptidoglycan layer so that effective interaction with the cytoplasmic membrane may absorb the polymers more deeply beyond the cell surface to result in good MICs values but insignificant zeta potential change. As for *E. coli*, the cationic DA100 polymer is likely to be constrained by the outer membrane which is typically a barrier particularly for large molecules. Thus, the polymer may aggregate outside the outer membrane to effect significant change to the measured zeta potential, but without causing severe disruption of the inner membrane to result in relatively poor antibacterial activity.

Figure 3.6 Effect of DA100 on the zeta potential change of *S. aureus* and *E. coli*.

The bacterial cytoplasmic membrane depolarization was also measured using the membrane-sensitive cyanine dye DiSC3(5) to show that DA100 kills bacteria by membrane disruption mechanism (For the Gram-negative *E. coli*, the bacteria treated with EDTA before adding DiSC3(5)). With addition of DA100 to the Gram-positive bacteria MRSA BAA40 (**Figure 3.7**), there is an immediate fluorescence intensity increase due to releasing of the dye because of the inner cell membrane depolarization. However, with the Gram-negative bacteria *E. coli*, DA100 almost didn't cause depolarization of the inner membrane while the positive control gramicidin S showed apparent increasing of fluorescence intensity. These results also lead to the hypothesis that DA100 is more likely dangling on the surface of outer membrane of *E. coli*, while it can interact with the cytoplasmic membrane of *S.*

aureus, resulting in the stronger depolarization effect of DA100 to the Gram-positive *S. aureus* compared with the Gram-negative *E. coli*.

Figure 3.7 Effect of DA100 on the membrane potential change of a) *S. aureus* and b) *E. coli*.

Figure 3.8 Biofilm inhibition of A100 and DA100 against (a) *E. coli* K12 and (b) MRSA BAA40. The data are averages of triplicates and the error bars indicate the standard deviations. “UC”: untreated control.

Moreover, DA100 and A100 have been examined by the inhibition ability of biofilm formation at MIC and sub-MICs by crystal violet staining assay. From **Figure 3.8a**, it is remark to see the DA100 copolymer inhibited the biofilm formation of *E. coli* K12 for most all concentrations tested and has a lower MBIC₅₀ (the lowest concentration at which at least 50% reduction in biomass of biofilms was measured compared to untreated biofilm samples) at 2 µg/mL. In contrast, the A100 showed gradual, concentration dependent inhibition of biofilm formation with MBIC₅₀ was around 128 µg/mL. The results showed that adding dextran to poly(AMPTMA) can decrease the biofilm formation. As for Gram-positive MRSA BAA40, both the A100 and DA100 polymers showed no effect on the biofilm formation of MRSA BAA40 at MIC or sub-MICs (**Figure 3.8b**).

Based on the studies of zeta potential (**Figure 3.6**) and DiSC3(5) (**Figure 3.7**), a possible mechanism of different biofilm inhibition ability of DA100 towards *E. coli* and *S. aureus* was shown in **Scheme 3.2**. During the biofilm formation, cationic DA100 may incorporated into biofilm and act differently with these two bacteria. As for Gram-negative *E. coli*, the weakly binding of the cationic polymers to the outer membrane of *E. coli*, leading to the antifouling dextran on the surface of *E. coli* to prevent the clustering of bacterial cells during the biofilm formation. As for the effect on *S. aureus*, the poor biofilm inhibition may be caused by more polymers interacting with the cytoplasmic membrane, leading to few or less polymers remaining on the peptidoglycan layer of *S. aureus*.

Scheme 3.2 Illustration of different binding mechanism of DA100 to *E. coli* and *S. aureus*.

Moreover, A100 and DA100 were also tested to study the preformed biofilm removal activity against Gram-negative strain *E. coli* and Gram-positive strain *S. aureus* using the MBEC™ assay. The results show that no clear biofilm reduction was found for both A100 and DA100 against these two bacteria (**Figure 3.9**). The incapability of the preformed biofilm removal of these cationic polymers may be due to the poor penetration ability of these polymers into EPS.^{22, 23}

Figure 3.9 CFU counting of biofilm Gram-negative *E. coli* after polymer treatment with (a) A100 and (b) DA100; CFU counting of biofilm Gram-positive *S. aureus* after polymer treatment with (c) A100 and (d) DA100. ns: not significant decrease. Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

3.4 Conclusions

In this chapter, I developed a new series of cationic polymers by applying SARA ATRP. The cationic DA100 played an important role in the antimicrobial activity against some clinic strains of Gram-positive *S. aureus*, including MRSA, with the MICs of 8-16 $\mu\text{g/mL}$; and also showed good biofilm inhibition against Gram-

negative *E. coli* without apparent antibacterial activity. The high selectivity is mainly attributed to the different binding interaction of the cationic polymers with the surface of different bacteria. Further, DA100 showed less cytotoxicity than A100 towards 3T3 cells and non-haemolytic. The biofilm inhibition results also demonstrated that the DA100 can prevent the biofilm formation of *E. coli* with efficacy much better than that of A100. However, both A100 and DA100 are incapable to remove the preformed biofilm of both Gram-negative and Gram-positive bacteria, which is highly possibly due to the poor penetration into biofilm. Therefore, further modification of the DA100 should be carried out to remove the preformed biofilm, which would be further discussed in **Chapter 4**.

3.5 Acknowledgements

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Chapter 4 Block Copolymer Nanoparticles Remove Biofilms of Drug-Resistant Gram-Positive Bacteria by Nanoscale Bacterial Debridement

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4.1 Introduction

Bacteria have developed resistance towards almost all classes of antibiotics, with serious consequences for anti-infection therapy. Further, bacterial infections often occur in biofilm form in which bacteria are protected by EPS.^{15, 16} Common antibiotics, which typically eradicate metabolically active planktonic bacteria, may be as much as 1000-fold less potent against biofilm bacteria.¹⁸⁸ The combination of multi-drug resistance and the protective character of biofilms is particularly worrisome from the perspective of therapy.

There has been much recent effort to develop new antibiofilm agents.¹⁸⁹ Small molecules,^{32, 37, 190} such as bromophenazine,¹⁹⁰ have been found to eradicate biofilm formed by Gram-positive bacteria such as *S. aureus*. AMPs tend to get trapped in anionic biofilms and also suffer enzymatic degradation in biofilms.^{46, 60, 191} A few AMPs such as IDR-1018 have shown efficacy for removal of pre-established biofilm

by downregulation of genes involved in biofilm formation, but this may be prone to resistance evolution.^{26, 192, 193} Small molecules and AMPs commonly have biocompatibility issues in terms of acute toxicity and/or hemolysis.^{44, 194} Surfactants¹⁹⁵ and surfactant-like molecules¹⁹⁶ have also shown the ability to remove biofilm. Cetyltrimethylammonium bromide (CTAB)¹⁹⁷, sodium dodecyl sulfate (SDS)¹⁹⁸ and phenol-soluble modulins (PSMs)¹⁹⁶ have shown antibiofilm effect. However, their hemolytic properties limit their applications.^{199, 200} NPs are an alternative class of antibiofilm agents¹⁰² and many metallic nanocomposites, such as Ag NPs,¹¹⁰ Au NPs,²⁰ magnetic iron oxide NPs^{201, 202} and other metal complex NPs,¹⁸ have been demonstrated to have antibiofilm effects. However, the toxicity of these metal/inorganic NPs remains a concern.²⁰³ Polymeric micelles which are themselves not effective in dispersing biofilm but can function as nanocarriers of antibiofilm agents have been shown to improve biofilm dispersal efficacy¹⁶¹ and may possess good biocompatibility.^{29, 148, 156, 158, 204} Again, these antibiofilm NPs remove biofilms through bactericidal action. There are few previous research reports on antibiofilm agents which are not antibacterial^{205, 206} and such non-bactericidal antibiofilm agents are of great interest because they are not affected by the problem of conventional antibiotics resistance.

Herein, in this chapter, I developed a novel polymeric NPs that can effectively remove biofilms of multi-drug resistant Gram-positive bacteria. The weakly amphiphilic cationic block copolymer of dextran and poly(AMPTMA-*co*-BMA) (hereafter called DA95B5) self-assembles into NPs with a thin polysaccharide shell and a cationic core. These NPs diffuse through biofilms of Gram-positive bacteria to

electrostatically complex with bacteria surfaces without killing the bacteria. Instead, the NPs cause the gradual removal of biofilms by weakening the attachment of the bacteria to the biofilm and exhibit biofilm removal efficacy comparable or superior to current standard antibiotics. Specifically, DA95B5 effectively removes biofilms of MRSA, VRE and also *Enterococcus faecalis* OG1RF which is implicated in catheter-associated infections. *In vivo* data (using a murine excisional wound model) also shows that DA95B5 solution when soaked into a hydrogel pad dressing can remove MRSA biofilm with efficacy better than vancomycin. The NPs are also non-hemolytic *in vitro* and have low *in vivo* cytotoxicity. This is the first report of polymeric NPs with a new biofilm removal mechanism, which we term “nanoscale bacterial debridement,” that is orthogonal to bactericidal activity and antibiotics resistance. This new class of agent has good Gram-positive biofilm removal efficacy and is as effective in biofilm removal of multi-drug resistant Gram-positive bacteria as is it for drug-sensitive strains.

4.2 Experimental Section

Materials

Dextran ($M_n=6000 \text{ g mol}^{-1}$), di-tert-butyl dicarbonate (BOC_2O , 98%), ethylene diamine (99%), 2-bromoisobutyrylbromide (98%), (3-acrylamidopropyl)trimethylammonium chloride solution 75 wt. % in H_2O , sodium cyano-borohydride (NaCNBH_3 , 98%), butyl methacrylate (BMA, 99%, contains 10 ppm monomethyl ether hydroquinone as inhibitor), copper (I) bromide (CuBr , 99%), copper (II) bromide (CuBr_2 , 99%), Ethyl α -bromoisobutyrate (EBiB, 98%), $\text{N,N,N',N'',N'''}\text{-pentamethyldiethylenetriamine}$ (PMDETA, 99%), dimethyl

sulfoxide (DMSO), 3,3'-dipropylthiadicarbocyanine iodide: DiSC3(5), and tetrahydrofuran (THF) were obtained from Sigma-Aldrich; it was stirred overnight with CaH_2 and distilled prior to use.

SARA ATRP of A95B5

33 mg EBiB (0.017 mmol) was dissolved in 2 mL DMSO followed by adding AMPTMA solution (75 wt. % in water, 1.615 mmol, 95 equiv.), BMA (0.085 mmol, 5 equiv.), 2.4 mg CuBr (0.017 mmol, 1.0 equiv.), 1.9 mg CuBr₂ (0.0085 mmol 0.5 equiv.), and 14.7 mg PMDETA (0.0085 mmol, 0.5 equiv.). Next, Cu (0) wire was added and then deoxygenated by purging with argon. Then the mixture was reacted at 50 °C in oil bath for 24 h. Then dialyzed against DI water with 6-8 kDa cutoff dialysis membrane. After dialysis the product was obtained after lyophilization.

SARA ATRP of DA95B5

In a Schlenk tube, 0.1 g Dextran-Br (0.017 mmol) was dissolved in 3 mL DMSO followed by adding AMPTMA solution (75 wt. % in water, 1.615 mmol, 95 equiv.), BMA (0.085 mmol, 5 equiv.), 2.4 mg CuBr (0.017 mmol, 1.0 equiv.), 1.9 mg CuBr₂ (0.0085 mmol 0.5 equiv.), and 14.7 mg PMDETA (0.0085 mmol, 0.5 equiv.). Next, Cu (0) wire was added and then deoxygenated by purging with argon. Then the mixture was reacted at 50 °C in oil bath for 24 h. Then dialyzed against DI water with 6-8 kDa cutoff dialysis membrane. After dialysis the product was obtained after lyophilization.

Characterization

The characterization of synthesized compounds was measured by ¹H NMR spectra (Bruker Avance II 300 MHz NMR Spectrometer). Gel permeation chromatography

(GPC) equipped with a refractive index detector to measure the molecular weights and polydispersity. The prepared samples (1 mg/mL) were injected into Water's GPC system equipped with two ultrahydrogel columns using acetate buffer (pH ~4.5) as the elute. Narrow distributed pullulan standards were used for the calibration curve. For surface tension, the polymer solution was measured by a Surface Tensiometer (DCAT 21, Data Physics Instruments GmbH, Germany) at 298.15 ± 0.1 K. To visualize the structure of DA95B5 NPs, TEM (Carl Zeiss Libra 120 plus, 120kV) was used.

Light Scattering Study of Copolymers

Prior to measurement, the polymer solutions were prepared in DI water or PBS followed by filtration against PES filter (0.45 μm). BI-200SM light scattering system (Brookhaven Instruments) was used to measure the hydrodynamic radius (R_h) with scattering angles from 30 to 150 degree and the radius of gyration (R_g) with scattering angles from 30 to 90 degrees. Mathematical analysis of the measured autocorrelation functions followed a published protocol.²⁰⁷

***In Vivo* Biocompatibility Studies**

All animal experiments were carried out in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes approved by the Ethics Committee of Union Hospital, Huazhong University of Science and Technology. Female BALB/c mice (6–8 weeks, 18–22 g) were selected randomly and divided into one control group and 2 treatment groups (n=6 in each group): DA100 and DA95B5. The control group was injected with 200 μL PBS and the treatment groups were injected with DA100 and DA95B5 at dosage of 10 mg/kg. Injection was given

intravenously through the tail vein. The liver and kidney function as well as balance of electrolytes were determined using Celercare M (MNCHIP, Tianjin).

Histological Examination

The mice were sacrificed 7 days after polymer injection and histological examination was performed on main organs including heart, lung, liver, spleen and kidney. Tissues were fixed in 10 % formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

***In Vivo* Mouse Model of MRSA Infection**

Female C57BL6 mice (Invivos, Singapore) aged 8 weeks were used in the *in vivo* excision wound model and experiments were performed according to protocols approved by the institutional animal care and usage committee (IACUC) of the Nanyang Technological University (protocol approval number IACUC A0362). All mice were housed on a 12-hour light-dark cycle at room temperature for one day prior to the experiment. Mice (n = 5 per group) were anesthetized using isoflurane and an excision wound was created on the dorsal area using a 5 mm diameter biopsy punch. 2.5 μ L MRSA BAA40 in PBS suspension (5×10^5 CFU/mL) was dispensed to wound site using a 10 μ L pipet and covered by Tegaderm (3M^{TM}) to prevent contamination. 24-hour after inoculation, Tegaderm film was removed and first treatment was applied by covering the wound site with polymer-soaked (2.5 mg/kg) hydrogel.²⁰⁸ A new layer of Tegaderm was applied to immobilize the hydrogel and prevent contamination. PBS soaked hydrogel as negative control and vancomycin-soaked (2.5 mg/kg) hydrogel as antibiotic control. Afterwards, mice were returned to their cages and rested for 4 hours. 2nd and 3rd treatments were subsequently

applied with 4-hour intervals. Mice were sacrificed using CO₂ 4 hour after last treatment and tissue samples were harvested using scalpel blade. Samples were further homogenized and plated on agar plates to determine CFU.

Analysis of Preformed Biofilm Assay

The MBEC assay^{209, 210} followed ASTM E2799-17. A Calgary device was inoculated with 150 µL of bacterial suspension (an approximate cell density of 10⁵ CFU/mL) in tryptic soy broth (TSB). The preformed biofilm was established at 37 °C for 24-48 hours. After incubation, the lid of Calgary device was then washed and transferred to a challenge plate which containing polymer solution with two-fold dilution with total volume of 200 µL. After incubated at room temperature for 2 hours, the lid was then again removed from the challenge plate and transferred to the recovery plates containing neutralizer. The recovery plate was sonicated for 30±5 min to remove and disaggregate the biofilm. The suspensions were diluted with PBS and plated on agar plates to determine CFU.

The crystal violet (CV) stain assay was conducted following a published report with minor modification.¹⁹ Briefly, 150 µL of the bacterial suspension in TSB (10⁵ CFU/mL) was added into 96-well plate. After 24 hours incubation at 37 °C, the preformed biofilms were washed with DI water and followed by adding different concentrations of polymer solution with a total volume of 200 µL in each well. After 2 hours treatment, the 96-well microplates were washed with DI water and stained with 100 µL of 0.1 % CV solution at room temperature for 15 minutes. After staining, the microplates were then washed with DI water and added 200 µL of 37 % acetic

acid to dissolve CV. Biofilm biomass reduction was recorded by measuring the OD₅₅₀ with a TECAN microplate reader.

TEM Study

Log phase bacteria (10^8 CFU/mL) were prepared 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 (Titan Krios transmission electron microscope, FEI Company) at National University Singapore Center for Bioimaging Science.

Fixing Biofilms for SEM

The assay was performed as described previously.²¹¹ The pegs from Calgary Biofilm Device were broken off and rinse in PBS to detach loosely-adherent bacteria. The pegs were then fixed in 2.5% glutaraldehyde at 4 °C for 16 h and washed in DI water for 10 min. The pegs were then dehydrated in 70% ethanol for 20 minutes and air dry for 24 h before specimen mounting and examination by SEM (JEOL JSM 6701F).

CLSM Study

MRSA BAA40 was cultured overnight in Luria Bertani (LB) broth in a shaking 37 °C incubator. Overnight culture was taken to dilute to 0.01 OD in 10 mL tryptic soy broth in a 50 mL Falcon tube. A sterile glass slide was gently added into the diluted culture and was taken to incubate static at 37 °C incubator. Next day, the glass slide was transferred using a sterile forcep to a falcon tube containing 12 mL of PBS with 128 µg/mL of the rhodamine-tagged DA95B5 NPs and this was incubated for 2 hours before imaging was taken. The cell images were captured using

the Zeiss LSM780 confocal laser scanning microscope (CLSM; Carl Zeiss, Jena, Germany) and LSM 780 ELYRA PS.1 superresolution structured illumination system (Carl Zeiss, Germany) with a 63× plan-apochromatic oil immersion objective lens (numerical aperture, 1.46). SYTO™ 9 green fluorescent and rhodamine were excited using 488 nm and 561 nm optically pumped semiconductor laser line for observation and data collection respectively. The captured images were further processed with the Zen 2011 software (Carl Zeiss, Germany) and Imaris software. To study the dynamics of biofilm dispersal effect of NPs. The MRSA BAA40 biofilm was co-cultured with 128 µg/mL NPs, and the time-lapse confocal microscopy was studied with CLSM. The images were acquired and analyzed using ZEN 2011 software.

Statistical analyses: were performed using either student's *t*-test or one-way analysis of Anova with Dunnett's correction wherever appropriate. P-values <0.05 were taken to be statistically significant.

4.3 Results and Discussions

In this chapter, hydrophobic monomer BMA was introduced into dextran-*block*-poly(AMPTMA) copolymer DA100. Therefore, three copolymers with different ratios of hydrophilic AMPTMA to hydrophobic BMA were synthesized via SARA ATRP. The three copolymers are (1) dextran-*block*-poly(AMPTMA(95%)-*co*-BMA (5%)) (DA95B5); (2) dextran-*block*-poly(AMPTMA(90%)-*co*-BMA (10%)) (DA90B10); and (3) dextran-*block*-poly(AMPTMA(80%)-*co*-BMA (20%)) (DA80B20). ¹HNMR and GPC data (**Appendix Figure A1**) indicated the successful synthesis of these copolymers.

The antibacterial activity of these copolymers was then tested against different bacterial strains. The results (**Appendix Table A1**) showed that there was a decreasing of bacterial killing ability by introducing small amount (5%) hydrophobic BMA into DA100 (**Table 3.2**), the MICs values of DA95B5 against *E. coli* and MRSA BAA40 was increased to as high as 512 µg/mL. Further, when introducing more amount of BMA, both DA90B10 and DA80B20 lost their killing efficacy against Gram-negative and Gram-positive bacteria with MICs values greater than 512 µg/mL, indicating the appearance of hydrophobic BMA has huge impact on the antibacterial activity of this series copolymers. Among them, DA95B5 showed less cytotoxicity towards 3T3 cells (**Appendix Figure A2**) with the bacterial killing ability at 512 µg/mL and it was selected as the optimized sample for further study accordingly.

Figure 4.1. ^1H NMR spectrum of A95B5 in D_2O .

Figure 4.2 ^1H NMR spectrum of DA95B5 in D_2O .

Table 4.1 GPC data of polymers.

Samples	Mn(GPC)	DPn/Mn(NMR)	PDI
Dextran(6k)-Br	5700	-	1.53
A100	9600	60/12300	1.52
DA100	15500	64/18800	1.57
A95B5	9800	-	1.60
DA95B5	9700	-	1.49

Figure 4.3 GPC traces for (a) dextran-br, (b) A100, (c) DA100, (d) A95B5, and (e) DA95B5.

Therefore, two more polymers investigated in this chapter were DA95B5 and its counterpart A95B5 which don't have dextran. The ^1H NMR spectra (**Figure 4.1-4.2**) and GPC (**Table 4.1 and Figure 4.3**) confirmed the successful syntheses of these polymers. Dynamic light scattering (DLS) (**Table 4.2**) was used to study four polymers, including A100 and DA100 discussed in Chapter 3. The analysis revealed that all the polymers, except DA95B5, existed in DI water as individual molecules with hydrodynamic radius (R_h) less than 10 nm at the concentration of 512 $\mu\text{g/mL}$. However, DA95B5 self-aggregated into NPs with R_h of 75.2 ± 3.1 nm and had a critical micelle concentration (CMC) of around 32 $\mu\text{g/mL}$, which was determined using pyrene as a fluorescent probe (**Figure 4.4**).

The R_g (radius of gyration) was also measured; the R_g/R_h ratio of DA95B5 NPs in DI water was around 0.4 (**Table 4.2**), indicating that the copolymer self-aggregated in DI water into core-shell NPs.²¹² The average diameter of DA95B5 NPs determined by TEM was found to be between 20 and 30 nm (**Figure 4.4**). The size of NPs determined by TEM was smaller than that by DLS, which might be attributed to the fully hydrated NPs during DLS measurement whereas dry and collapsed NPs from TEM.²¹³ It was also found that DA95B5, which contains the hydrophobic BMA constituent, reduced the solution surface tension (**Table 4.2**) from around 70 mN/m to about 45 mN/m. The zeta potential values of all the (co)polymers in DI water were in the range 36 to 41 mV.

Figure 4.4 (a) The intensity ratio I_3/I_1 in the fluorescence excitation spectra of pyrene as a function of concentration of DA95B5 solution (in DI water); (b) TEM image of micelles formed by DA95B5, scale bar=100 nm.

Table 4.2 Particle size, zeta potential and surface tension of polymers in DI water.

Polymers	R_g (nm)	R_h (nm)	R_g/R_h	Zeta potential (mV)	Surface tension(mN/m)
Dextran	none	1.1 ± 0.1	none	1.1 ± 1.5	62.7 ± 1.9
A100	none	0.9 ± 0.1	none	35.3 ± 12.3	50.6 ± 0.6
DA100	none	1.2 ± 0.7	none	41.3 ± 4.0	54.7 ± 1.2
A95B5	none	5.3 ± 2.1	none	36.2 ± 2.9	42.1 ± 1.2
DA95B5	32.0 ± 0.8	75.2 ± 3.1	0.426	39.4 ± 6.7	45.1 ± 1.8

MICs values of polymers against both Gram-positive and Gram-negative bacteria were shown in **Table 4.3**. The polymers A100 and DA100, as mentioned in Chapter 3, had excellent antimicrobial activity (8-16 µg/mL) against Gram-positive bacteria (*S. aureus*, including MRSA (BAA40 and USA300)), moderate efficacy (128-512 µg/mL) against the Gram-negative species *E. coli* and poor antibacterial activity against *E. faecalis* strains (VRE and OG1RF). For A95B5, which contains a small proportion of the hydrophobic BMA, the antibacterial activity against all the Gram-positive bacteria (*S. aureus*, MRSA, VRE and OG1RF) was similar to that of A100 or DA100, but the killing of *E. coli* was significantly improved (MIC: 32-64 µg/mL). However, the terpolymer DA95B5 had much higher MICs (≥ 512 µg/mL) against all of the planktonic pathogens tested.

Table 4.3 Biological properties of polymers and reference AMPs: minimum inhibitory concentrations (MICs: µg/mL), hemolytic concentration for 10% red blood cell lysis (HC₁₀, µg/mL) and the 50% inhibitory concentration with 3T3 cells (IC₅₀, µg/mL). “n.d.” indicates not determined.

	MIC: µg/mL							HC ₁₀ (µg/mL) RBC	IC ₅₀ (µg/mL) 3T3
	<i>S. aureus</i> (MRSA BAA 40)	<i>S. aureus</i> (ATC C 29213)	<i>S. aureus</i> (USA3 00)	V583	<i>E. faecalis</i> OG1R F	<i>E. coli</i> (ATC C 8739)	<i>E. coli</i> K1 2		
A100	8-16	16	8-16	>512	>512	128	256	>20000	65.2±13.2
DA100	8-16	16	8-16	>512	>512	128	256	>20000	141.7±17.4
A95B5	16	16	16	>512	>512	32	64	>20000	84.3±10.8
DA95B5	512	512	>512	>512	>512	512	512	>20000	194.7±18.1
Magainin 2	>512	>512	>512	>512	>512	64	64	>500	n.d.
Melittin	8	8	8	16	16	32	32	<10	n.d.

Using the MBEC™ assay based on ASTM E2799-17,^{209, 210} I observed that DA95B5 effectively reduced bacteria counts in preformed biofilms of Gram-positive bacteria (**Figure 4.5 and Table 4.4**). I studied the effect of DA95B5 copolymer on preformed biofilms of five multi-drug resistant/clinically relevant Gram-positive bacteria (MRSA BAA40, MRSA USA300, MRSA KKH5, VRE and *E. faecalis* OG1RF) and one drug-sensitive Gram-positive strain (*S. aureus* ATCC29213). DA95B5 showed reduction in cell counts of biofilm bacteria of all Gram-positive bacteria tested in a dose-dependent manner with efficacy much better than or similar to that of current standard antibiotics. For MRSA BAA40, DA95B5 reduced the biofilm bacterial cell counts by up to 2.0 log reduction after a single polymer treatment at 32 µg/mL; these reductions were higher than those of first-line MRSA antibiotics (oxacillin, doxycycline and linezolid) as well as that of the last resort antibiotic vancomycin.

Table 4.4 Log reduction of 5 multi-drug resistant/clinically relevant Gram-positive bacterial biofilm treated by DA95B5 and standard antibiotics.

	<i>Staphylococcus aureus</i>			<i>Enterococcus faecalis</i>	
	MRSA BAA40 (32 µg/mL)	MRSA USA300 (128 µg/mL)	MRSA KKH5 ^a (128 µg/mL)	V583 (128 µg/mL)	OG1RF (128 µg/mL)
DA95B5	2.0(* p ≤ 0.05)	1.1(* p ≤ 0.05)	1.7(* p ≤ 0.05)	0.8 (* p ≤ 0.05)	0.8 (* p ≤ 0.05)
Oxacillin	1.0(* p ≤ 0.05)	0.3(p=0.57)	1.6(* p ≤ 0.05)	n.d.	n.d.
Doxycycline	-0.3(P=0.3)	0.2(p=0.71)	0.7(* p ≤ 0.05)	n.d.	n.d.
Linezolid	0.9(* p ≤ 0.05)	0.6(p=0.19)	1.2(* p ≤ 0.05)	0.7(* p ≤ 0.05)	0.3(* p ≤ 0.05)
Vancomycin	0.3(P=0.18)	0.2(p=0.86)	0.7(* p ≤ 0.05)	n.d.	0.2(p=0.06)
Ampicillin	n.d.	n.d.	n.d.	1.8(* p ≤ 0.05)	0.8(* p ≤ 0.05)
Nitrofurantoin	n.d.	n.d.	n.d.	0.1(p=0.50)	0.3 (* p ≤ 0.05)

^a MRSA KKH5 provided by KK women's and children's hospital, Singapore. “n.d.” indicates not determined as this antibiotic is not used for the treatment of that bacteria. P ≤ 0.05 to be considered significant using student's *t*-test.

Figure 4.5 (a) Biofilm removal by DA95B5 measured by MBEC™ assay according to ASTM E2799-17. Viable Gram-positive bacterial counts of a)i MRSA BAA40, a)ii VRE and a)iii OG1RF on each microtiter plate peg after 2h treatment with DA95B5 compared with the standard antibiotics (Linezolid: yellow; Vancomycin: purple; Oxacillin: blue, Doxycycline: red, Ampicillin: grey, Nitrofurantoin: orange); Data are presented as mean $\pm$ standard deviation and represent three independent experiments. (b) Representative FESEM images of Gram-positive bacteria b)i MRSA BAA40, b)ii VRE and b)iii

OG1RF biofilms on pegs of the MBEC biofilm inoculator before and after DA95B5 treatment (with 128 µg/mL). Scale bar=1 µm. (c) Scheme of *in vivo* study of antibiofilm activity of DA95B5/vancomycin soaked hydrogel against MRSA BAA40 biofilm in an established murine excision wound model. (d) Log CFU per wound from hydrogel alone, DA95B5-soaked (2.5 mg/kg) and vancomycin-soaked (2.5 mg/kg) hydrogels. Each type of hydrogels were applied at three times at 4-hours intervals before plating for CFU determination on agar plates. *** $p \leq 0.001$ and **** $p \leq 0.0001$ by two-tailed Student's *t*-test.

DA95B5 also reduced the biofilm cell counts of other *S. aureus* strains: it achieved generally higher log reductions (*i.e.* 1.1, 1.7 and 1.2) against MRSA USA300, MRSA KKH5 and ATCC29213 biofilm bacteria respectively compared to those of standard antibiotics (**Figure 4.6**). Against VRE biofilm bacteria, DA95B5 achieved 2.5 log reduction, albeit with a higher concentration of 512 µg/mL, which is not achievable by any of the standard antibiotics investigated that only achieved maximum log reduction of 1.3 with ampicillin antibiotic. With a lower concentration of 128 µg/mL, DA95B5 showed 0.8 log reduction of VRE biofilm bacteria which was still better than the standard antibiotics linezolid and nitrofurantoin (with 0.7 and 0.1 log reductions, respectively) but worse off compared to ampicillin (with 1.8 log reduction). With the clinically relevant bacteria OG1RF, DA95B5 (128 µg/mL) reduced the biofilm bacteria by 0.8 log reduction, which was superior or comparable to the current standard antibiotics (0.2 to 0.8 log reduction) (**Table 4.4**).

Figure 4.6 Biofilm removal by DA95B5 measured by MBEC™ assay according to ASTM E2799-17. Viable Gram-positive bacterial counts of (a) MRSA BAA40, (b) USA300, (c) MRSA KKH5, (d) ATCC29213, (e) VRE and (f) OG1RF on each microtiter plate peg after 2h treatment with DA95B5 compared with the standard antibiotics (Linezolid: yellow; Vancomycin: purple; Oxacillin: blue, Doxycycline: red, Ampicillin: grey, Nitrofurantoin: orange); Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

The FESEM images (**Figure 4.5b**) corroborated that the cell densities of Gram-positive biofilm bacteria clearly declined after treatment with a single dose (128 $\mu\text{g/mL}$) of DA95B5. However, DA95B5 showed no/poor reduction of biofilm bacteria of the two Gram-negative strains (*E. coli* ATCC8739 and *E. coli* K12) tested (**Figure 4.7**).

I also investigated the efficacy of DA95B5 in removing more matured biofilms. The results showed that DA95B5 at the same concentration (*i.e.* 32 $\mu\text{g/mL}$) can also remove the longer-term 3-day biofilm by around 2.0 log reduction, so that its efficacy was much higher than that of vancomycin (1.0 log reduction). With even longer-term (7-day) biofilm, DA95B5 at higher concentration (*i.e.* 512 $\mu\text{g/mL}$) reduced the biofilm by 1.7 log reduction as opposed to around 0.1 log reduction with vancomycin, again corroborating the superior efficacy of DA95B5 in MRSA BAA40 biofilm removal (**Figure 4.8**).

Figure 4.7 Biofilm removal by DA95B5 tested by MBEC™ assay according to ASTM E2799-17. Viable Gram-negative bacterial counts (a) *E. coli* ATCC8739 and (b) K12 on each microtiter plate peg after 2h treatment with DA95B5 compared with the standard antibiotic rifampicin. Data are

presented as mean $\pm$ standard deviation and represent three independent experiments.

Figure 4.8 Removal of longer-day biofilms by DA95B5 tested by MBEC™ assay according to ASTM E2799-17. (a) 3-day biofilm and (b) 7-day biofilm removal. Viable MRSA BAA40 biofilm bacterial counts on each microtiter plate peg after 2h treatment with DA95B5 compared with antibiotic vancomycin. Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

The superior *in vivo* antibiofilm efficacy of a hydrogel dressing soaked with DA95B5 solution was further demonstrated using a murine excisional wound model (**Figure 4.5c**). An excisional wound was created and 10^3 CFU MRSA BAA40 bacteria was inoculated to the wound site. After 24-hour development, the bacteria in each wound greatly multiplied to 10^8 ~ 10^9 CFU, establishing severely infected wounds with biofilms. A porous hydrogel²⁰⁸ dressing soaked with DA95B5 solution (2.5 mg/kg) was applied three times to fully cover the wound site, with 4-hour interval between each treatment. PBS and vancomycin-soaked (2.5 mg/kg) hydrogels were used as infection and antibiotic controls respectively. Based on the

results in **Figure 4.5d**, the DA95B5 treatment effectively reduced the biofilm bacteria on the wound site ($p < 0.0001$), achieving a log reduction of 3.6 of total bacteria count. Vancomycin treatment achieved only 1.7 log reduction of biofilm bacteria burden, which was significantly less effective compared with our polymer ($p < 0.001$).

The penetration of DA95B5 (and the control DA100) into MRSA BAA40 biofilm was then investigated by time-lapse CLSM using rhodamine labelled polymers. To quantify the penetration profiles of the NPs/polymer, z-stack confocal imaging (with image analysis done by ImageJ software) was used to determine the locations of NPs. As shown in **Figure 4.9a**, DA95B5 can penetrate quickly into the MRSA BAA40 biofilm within 5 minutes, probably because of electrostatic interactions. As the lapsed time goes beyond 5 mins (say 10 to 15 mins), the highest red fluorescence intensity began to shift from 5.6 μm to 6.8 μm , indicating the NPs probably then diffuse into the deeper layers of the biofilm. In contrast, the control polymer DA100 (**Figure 4.9b**) showed almost no diffusion in the same period of time although after longer time (30 mins), it also diffuses into deeper layers of biofilm. Hence, the self-assembled DA95B5 NP with the hydrophilic corona can diffuse faster into the Gram-positive MRSA biofilm than DA100 polymer that exists as individual molecules.

Longer time-lapse confocal microscopy (**Figure 4.9c**) shows that the MRSA BAA40 biofilm can be dispersed by DA95B5 (128 $\mu\text{g/mL}$, ≥ 60 min) as indicated by decrease of green fluorescent labelled MRSA BAA40 bacteria signals, which corroborates the lower CFU count results of the MBEC™ assay (**Figure 4.5a**). However, as for Gram-positive *E. coli* ATCC8739, the penetration profile of

DA95B5 into biofilm shows that there is no diffusion and accumulation of NPs into the *E. coli* ATCC8739 biofilm even after 60 min incubation (**Appendix Figure A3**), which might be assist in the explanation of no/poor reduction of biofilm bacteria of DA95B5 against the Gram-negative strains (**Figure 4.7**).

Figure 4.9 Penetration profiles of polymers at different time points. (a) Penetration profile of DA95B5; (b) Penetration profile of DA100. The x-axis is the depth of penetration of biofilms, where 0 μm represents the top layer of biofilm and $\sim 6.8 \mu\text{m}$ (represented by dashed vertical line) the bottommost layer of biofilm. The y-axis is normalized intensity of red channels. (c) Time-

lapse 3D confocal images of MRSA BAA40 biofilms treated by DA95B5 at 128 $\mu\text{g/mL}$ with incubation time: 0 min, 5 min, 10 min, 30 min, 60 min and 120 min, showing the dispersal of biofilm.

I further titrated the representative Gram-positive bacteria (MRSA BAA40, VRE and OG1RF) with increasing concentrations of DA95B5 and measured the zeta potential of the treated bacteria. The zeta potential of the treated Gram-positive bacteria changed from negative to positive with increasing concentrations of DA95B5 (**Figure 4.10a**), indicating binding of the cationic polymers with bacterial surfaces. However, measurement of the bacterial cytoplasmic membrane depolarization using the membrane-potential-sensitive cyanine dye DiSC3(5) (**Figure 4.10b**) showed that DA95B5 did not cause cytoplasmic membrane depolarization in these Gram-positive bacteria, indicating poor membrane penetration ability of DA95B5. We also studied the interaction of DA95B5 NPs with MRSA BAA40 bacteria using cryo-TEM (**Figure 4.10c**). These images show that DA95B5 accumulated around the surface of bacteria as NPs without disassembly, and the bacterial cell wall remained intact. Overall, all the results indicate that DA95B5 NPs coat the surfaces of Gram-positive bacteria but do not penetrate into the cytoplasmic membrane; these behaviors confer its non-bactericidal properties.

Figure 4.10 Effect of DA95B5 on the properties of three Gram-positive strains, specifically: (a) zeta potential after incubation with DA95B5. (b) membrane potential change, assessed by DiSC3(5) fluorescence, of b)i MRSA BAA40; b)ii VRE and b)iii OG1RF after DA95B5 treatment. Polymer added at first arrow; 100 μg/mL Gramicidin S added at second arrow as positive control to indicate 100% membrane depolarization. The polymer did not depolarize cytoplasmic membrane. (c) cryo-TEM images of the c)i MRSA BAA40 bacteria, c)ii DA95B5 NPs in PBS buffer and c)iii the location of DA95B5 NPs in the MRSA BAA40 bacteria. The arrows denote NPs coated onto bacteria surface. Scale bars are 100 nm.

In addition to its excellent antibiofilm activity, DA95B5 was exceptionally non-hemolytic, with concentrations for 10% hemolysis of human red blood cells ($HC_{10} > 20,000 \mu\text{g/mL}$) much higher than those of most AMPs (**Table 4.3**). In *in vitro* acute toxicity testing, the respective 50% inhibitory concentrations (IC_{50}) of DA95B5 towards various mammalian cells were $194 \mu\text{g/mL}$ (3T3), $2200 \mu\text{g/mL}$ (human foreskin fibroblasts, HFF), and $1461 \mu\text{g/mL}$ (hypertrophic scar-derived fibroblasts, HSF) (**Figure 4.11**). By comparing the IC_{50} of different cell lines, it is clearly to find that 3T3 cells are more sensitive to DA95B5, and this phenomenon might be attributed to the differences of morphology,²¹⁴ innate nature and capability of these different cell lines.²¹⁵ Further, live/dead viability assay was used to determine the membrane integrity of 3T3 cells after DA95B5 treatment. The live cells with intact membrane were stained by green-fluorescent calcein-AM, while the dead cells with compromised membrane showing red- fluorescent by staining EthD-1. The results (**Appendix Figure A4**) show that there was no apparent membrane damage (red fluorescence) caused by the treatment of DA95B5 at concentrations of $100 \mu\text{g/mL}$ and $200 \mu\text{g/mL}$ (close to IC_{50}). However, there was a reduction of live cell counts (green fluorescence) after treatment of DA95B5 compared to untreated control, which might be attributed to the effect of DA95B5 on the cells' attachment and further inhibit the proliferation of 3T3 cells.

Nevertheless, the *in vitro* biocompatibility results with 3T3 fibroblasts (**Figure 4.11b**) show that DA95B5 at effective biofilm dispersal concentration ($32\text{--}128 \mu\text{g/mL}$, **Table 4.4**) are non-toxic (with survival of 3T3 fibroblasts around 70%) and indicate that the addition of dextran improves the biocompatibility so that DA95B5

is less toxic than A95B5 (and DA100 is less toxic than A100). Therefore, DA95B5 would be a good candidate to treat wound infections with appropriate dosages. Overall, DA95B5 showed excellent *in vitro* biocompatibility possibly due to the dextran component and the core/shell NP morphology.

Figure 4.11 (a) Hemolytic activity of A100, DA100, A95B5 and DA95B5; (b) Various mammalian cells (HFF, HSF, and 3T3) viability of DA95B5. The data are average of triplicates and the error bars indicate the standard deviations.

In vivo intravenous administration tests on mice with DA95B5 at 10 mg/kg in a murine model showed no significant acute toxicity; the biomarkers of liver and kidney (*i.e.* alanine transaminase, aspartate transaminase, total bilirubin, creatinine and urea nitrogen) and electrolyte levels (*i.e.* sodium and potassium ions) in blood samples remained unchanged 24 h and 7 days after injection (**Table 4.5**). Further, histological assessment of vital organs (**Figure 4.12**) showed no apparent histopathological abnormalities or lesions in heart liver, kidney, lung and spleen of polymer-treated animals.

Figure 4.12 Histological images of main organs of mice at 7 days after polymers (DA100 and DA95B5) injection. (a) heart, (b) kidney, (c) liver, (d) spleen and (e) lung. Scale bar=50 μm .

Table 4.5 Effect of DA95B5 after 1 day and 7 days' treatment on liver and kidney functions and polyelectrolyte balance in the blood.

	Control (no polymer)	1 day challenge	7 days challenge
ALT (U L^{-1})	41.3 $\pm$ 2.1	39.3 $\pm$ 5.1(p=0.390)	40.0 $\pm$ 0.7(p=0.063)
AST (U L^{-1})	100.5 $\pm$ 11.6	94.2 $\pm$ 11.0(p=0.355)	96.3 $\pm$ 9.8(p=0.517)
TBIL ($\mu\text{mol L}^{-1}$)	3.8 $\pm$ 0.21	3.8 $\pm$ 0.4(p=0.929)	3.6 $\pm$ 0.5(p=0.459)
Creatinine ($\mu\text{mol L}^{-1}$)	17.5 $\pm$ 1.1	18.2 $\pm$ 1.5(p=0.388)	16.8 $\pm$ 2.6(p=0.578)
Urea nitrogen (mmol L^{-1})	9.8 $\pm$ 1.0	9.9 $\pm$ 1.0(p=0.909)	8.8 $\pm$ 0.8(p=0.106)
Potassium ion (mmol L^{-1})	5.1 $\pm$ 0.2	5.1 $\pm$ 0.2(p=0.742)	5.0 $\pm$ 0.4(p=0.539)
Sodium ion (mmol L^{-1})	148.3 $\pm$ 2.7	146.3 $\pm$ 6.4(p=0.498)	149.3 $\pm$ 3.9(p=0.617)

Note: ALT: alanine transaminase; AST: aspartate transaminase; TBIL: total bilirubin, $p < 0.05$ to be considered significant using p test.

Surprisingly, the addition of a small amount of the third monomer (BMA) into DA95B5 makes it non-bactericidal, but confers good preformed biofilm removal efficacy (**Table 4.4**) which was not observed in any other polymers (A100, DA100 and A95B5) (**Figure 4.13**).

Figure 4.13 CFU counting of biofilm bacteria (*S. aureus* ATCC29213) after polymer treatment tested by MBEC™ assay according to ASTM E2799-17. Viable bacterial counts of each peg after 2h treatment with 4 polymers against Gram-positive strain *S. aureus* ATCC29213. ns: not significant decrease. * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$; Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

TEM and DLS studies show that only DA95B5 copolymer forms NPs in solution. The positively charged (as indicated by the zeta potential measurements) DA95B5 NPs strongly associate with bacterial surfaces by electrostatic interaction, and physically interposes between the bacteria and the surrounding EPS matrix. The

cryo-TEM images show that DA95B5 retains its particulate morphology around the bacteria. The highly hydrophilic character of the dextran corona of DA95B5 particles around the bacteria would promote solvation of the bacteria/NP complex as well as its detachment from the biofilm (**Scheme 4.1**), resulting in biofilm dispersion.

Scheme 4.1 Mechanism of preformed biofilm removal by DA95B5 NPs (green: dextran; light blue: poly(AMPTMA-co-BMA)).

This mechanism is reminiscent of the process by which bacteria in mature biofilms can self-mobilize and detach through production of surfactant molecules.^{216, 217} This combination of events – NP complexation followed by solvation of the bacteria/NP complex -- by the nanostructured DA95B5 results in the detachment or “debridement” of individual bacteria from the biofilm matrix and consequent dispersal of the biofilm. This is a new mechanism of biofilm removal, which we term “nanoscale bacterial debridement”.

Various other reports of antibiofilm agents typically focus on inhibition of biofilm formation;²¹⁸ hence, removal of established biofilm remains a challenging problem. Although antibiotics such as vancomycin have much lower *in vitro* MIC values compared with our polymer, they are significantly less effective against established biofilm in the *in vitro* and *in vivo* models. Removal of established biofilm is highly clinically relevant since by the time of diagnosis, infections have typically progressed to the biofilm state. Most other agents that target biofilm bacteria do so by exerting anti-bacterial effects which, depending on the agents, may entail associated toxicities or vulnerability to existing or future resistance mechanisms.^{44,}
¹⁹¹ Unlike the killing effect of vancomycin, our novel copolymer NPs exert preformed biofilm removal effect by physically interposing themselves between the bacteria and the EPS to enhance the solvation of the bacteria/NP complex. This biofilm removal mechanism – nanoscale bacterial debridement -- is orthogonal to antibiotics resistance so that it can remove biofilms of MRSA, VRE, *etc.* that are invulnerable to many current standard antibiotics. Our *in vivo* wound model is clinically significant since MRSA is one of the most common pathogens in skin and soft tissue infections (SSTIs).²¹⁹ It is hypothesized that the observed *in vivo* biofilm removal was achieved synergistically by the DA95B5 polymer with the porous hydrogel: DA95B5 could diffuse from the hydrogel into the wound site to disperse the biofilm bacteria, and the slough bacteria released from the biofilm were subsequently absorbed by the porous hydrogel, preventing recurrent colonization or spreading of infection. The effective *in vivo* biofilm eradication makes our polymer an excellent candidate for clinical applications such as wound dressings or

disinfectant rinse for wounds and biomedical devices to eradicate biofilm-associated MRSA.

4.4 Conclusions

In conclusion, the amphiphilic block copolymer DA95B5 effectively removes preformed biofilms of multi-drug resistant Gram-positive bacteria by a new mechanism: nanoscale bacterial debridement. Specifically, DA95B5 removes the biofilms of multi-drug resistant/clinically relevant MRSA, VRE and OG1RF much better than/or comparable to current standard antibiotics. DA95B5 self-assembles into NPs which can diffuse through Gram-positive biofilm to attach to bacterial surfaces. The DA95B5 NPs dextran corona probably enhances the bacterial/NP complex solubility to increase bacterial detachment from the biofilm, resulting in reduction of biofilm biomass. The bacterial debridement mechanism is orthogonal to antibiotics resistance so that it removes biofilms of drug-resistant strains as effectively as those of drug-sensitive strains. *In vivo* data (using a murine excisional wound model) also shows that DA95B5 can effectively disperse MRSA biofilm with log reduction up to 3.6, which is significantly better than the efficacy of the last resort antibiotic vancomycin (1.7 log reduction). Further, DA95B5 is non-hemolytic *in vitro* and has negligible *in vivo* acute toxicity in a murine intravenous model at the dosage of 10 mg/kg. This novel biofilm removal approach demonstrates a powerful approach towards eradication of multi-drug resistant biofilm which can be employed in wound dressings or disinfectant rinse for wound treatment or prevention of biomedical devices-associated infections.

4.5 Acknowledgements

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Chapter 5 Biguanide-Derived Polymeric Nanoparticles for Eradicating MRSA Biofilm in a Murine Model

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5.1 Introduction

MRSA is a leading cause of morbidity and mortality due to infection.²²⁰ According to the US Centers for Disease Control and Prevention, in 2017, nearly 120,000 people suffered bloodstream infections caused by *Staphylococcus aureus* and about 1/6th of these infections were fatal.^{220, 221} MRSA is resistant to many beta-lactam antibiotics such as methicillin and oxacillin, as well as other major classes of antibiotics including macrolides and some fluoroquinolones. MRSA is notorious for causing recalcitrant chronic wound infections and is the most common pathogen in skin and soft tissue infections.²²² The ability of MRSA to develop robust biofilm on wound sites further complicates treatment, since biofilm bacteria are 10 to 1000 times more resistant than planktonic state bacteria to conventional antibiotics.^{20, 21} Once a biofilm is formed, its three-dimensional matrix of EPSs can protect the biofilm cells from antibacterial agents and immune clearance.¹⁵ If untreated, MRSA can disseminate from the skin to blood to cause life-threatening sepsis.²²³ New drugs

and technologies are urgently needed to eradicate difficult-to-treat biofilm infections, especially those developed by multidrug-resistant bacteria such as MRSA.

Cationic polymers have been studied as potential candidates to fight biofilm bacteria only in the past few years,^{94, 224, 225} although they have been widely used to combat planktonic bacteria, which are much easier to eradicate. Efforts have been made to develop novel antibiofilm agents, such as polysaccharide-based cationic polymer,^{85, 226} polypeptides,⁹⁴ cationic polyacrylates,^{87, 89, 227} and polycarbonate.²²⁴ Although these synthetic polymers show good antibacterial and/or antibiofilm efficacy, unselective toxicities and high raw materials cost have impeded their clinical application.⁶²

In the effort to develop next-generation antibiofilm agents to meet both efficacy and biocompatibility requirements, nanotechnology has attracted much attention.^{23,}
¹³⁰ The size, surface morphology and charge of NPs can be tuned to enhance penetration into the biofilm matrix.^{20, 156, 228, 229} Moreover, polymeric nanocarriers can serve as drug delivery systems with controllable release, through pH-sensitive mechanisms, of antibacterial cargoes upon interaction with the biofilm matrix.^{29, 30} Some other NPs act as antibiofilm agents in photodynamic therapy.^{20, 31} Nevertheless, there are still some limitations of current antibiofilm NPs. For example, many of these NPs are made from metals such as silver,^{110, 111, 230} gold²⁸ and iron,^{201,}
²³¹ which has raised concerns about their toxicities²³⁰ and resistance development.¹⁰⁵ In our previous study, we reported a novel NP system made of dextran-*block*-poly(AMPTMA-*co*-BMA) which has remarkably low toxicity and excellent biofilm dispersal ability via a mechanism we called “nanoscale bacterial debridement”.²³²

However, though this NP system effectively removes biofilm, it does not kill the dispersed bacteria – *i.e.* it is not intrinsically antibacterial. This is a drawback to potential clinical application of the material as the dispersed live bacteria may colonize other sites.

Biguanide-based compounds have been studied as small antibiofilm molecules. For instance, biguanide iridium (III) complex showed killing of mature biofilm bacteria of Gram-positive VRE with a mechanism of action possibly due to intracellular biguanide inhibition of some protein bio-synthesis.²³³ Further, some guanidine and biguanide modified norspermidines also exhibited good biofilm inhibition and preformed biofilm disruption activity against Gram-positive *S. aureus* by targeting the EPS of biofilm.³⁵ However, the *in vitro* and *in vivo* toxicities to mammalian cells of biguanide metal complexes and biguanidylated polyamines derived from norspermidine have yet to be determined so that the potential for development toward clinical applications of these compounds is unknown.

Herein, we reported a new series of polyphenol-assisted polymeric NPs with biofilm eradication ability by killing the bacteria inside biofilm as well as excellent *in vitro* and *in vivo* biocompatibility. Based on good antibacterial and antibiofilm effects of the biguanide-related compounds,^{35, 112, 233} we synthesized a novel biguanide-based PMET from linear polyethyleneimine (PEI, 5000 Da) via a facile one-step reaction (**Scheme 5.1a**).²³⁴ Moreover, the *in vitro* data showed that PMET is active against various *S. aureus* strains, including both community-associated and hospital-associated MRSA, with minimum inhibitory concentration of 8-16 µg/mL. However, the *in vitro* antibiofilm assay using only PMET alone showed limited

efficacy towards biofilm bacteria counts reduction even at high concentrations (128-512 $\mu\text{g/mL}$), possibly because of the poor biofilm penetration ability of neat PMET.

To further improve the biofilm eradication efficacy, TA and F-127 are introduced to form a polymeric NP system. The antibiofilm NPs consists of three main components: PMET, TA, and Pluronic® F-127 (**Scheme 5.1b**). TA, generally recognized as a safe (GRAS) compound by FDA, is a hydroxyl-rich polyphenol and can serve as building blocks in many supramolecular chemistry due to its ability to form hydrogen bond and hydrophobically interact with multiple macromolecules.²³⁵ However, these TA/PMET NPs (thereafter called TP NPs) will precipitate out from aqueous solution due to its poor colloidal stability. We then further introduced another FDA approved surfactant F-127, which has been reported to interact with TA by hydrogen-bonding.²³⁶ The F-127/TA/PMET combination can form stable NPs with well-defined structures (thereafter called FTP NPs). Compared with PMET, the FTP NPs showed improved biocompatibility towards mammalian cells *in vitro*, and enhanced biofilm eradication efficacy against MRSA USA300 biofilm both *in vitro* and *in vivo*. Further, the *in vivo* toxicity studies demonstrated that the FTP NPs introduced via the intravenous route induce no acute toxicity to mice without any apparently body weight loss and change of blood biomarkers.

5.2 Experimental Section

Materials

Linear polyethyleneimine (PEI, $M_n=5000$ Da), dicyandiamide (99%), tannic acid, Pluronic® F-127, hydrochloric acid (37%), dimethyl sulfoxide (DMSO), crystal violet (CV), 3,3'-dipropylthiadicarbocyanine iodide: DiSC3(5), dulbecco's

modified eagle medium (DMEM), trypsin-EDTA, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl2H-tetrazolium bromide (MTT), glutaraldehyde, and Triton X-100 were purchased from Sigma-Aldrich and used as received.

Preparation and Characterization of PMET

PMET was synthesized following a published protocol.²³⁴ Briefly, linear PEI (0.2 g, 0.04 mmol) was reacted with dicyandiamide (2.0 g, 23.7 mmol) in 10 mL 1M HCl at 100 °C for 48 h. After reaction, the mixture was filtered and the filtrate was titrated with 2 M NaOH to pH=10 and then dialyzed against DI water with 3.5 kDa cutoff dialysis membrane. After dialysis the product was obtained after lyophilization. The characterization of PMET was measured by ¹H NMR spectra (Bruker Avance II 300MHz NMR Spectrometer). Gel permeation chromatography (GPC) equipped with a refractive index detector to measure the molecular weights and polydispersity. The prepared samples (1 mg/mL) were injected into Water's GPC system equipped with two ultrahydrogel columns using acetate buffer (pH ~4.5) as the elute. Narrow distributed pullulan standards were used for the calibration curve.

Preparation of TP and FTP NPs

To prepare TP NPs, 50 µL TA (20 mg/mL) solution were dropped quickly into 1 mL PMET (10 mg/mL) aqueous solution under stirring at 1000 rpm for 10 mins. To prepare FTP NPs, a mixture solution (1:1, v/v) of 50 µL TA (20 mg/mL) and F-127 (20 mg/mL) solution were first prepared. Subsequently, the mixture was quickly dropped into 1 mL PMET (10 mg/mL) aqueous solution under stirring at 1000 rpm and further maintained stirring for 10 mins. The final products were dialyzed against DI water with 12-14 kDa cutoff dialysis membrane and obtained after lyophilization.

The particle size and zeta potential were determined by Malvern Nano-ZS Particle Sizer (Malvern Instruments, Malvern, U.K.). Transmission electron microscope (TEM) images of NPs were acquired with JEOL JEM-2100Plus Electron Microscope (Tokyo, Japan).

***In Vitro* Antibiofilm Assays**

The MBEC assay^{209, 210} followed ASTM E2799-17. A Calgary device was inoculated with 150 μ L of bacterial suspension (an approximate cell density of 10^5 CFU/mL) in tryptic soy broth (TSB). The preformed biofilm was established at 37 °C for 24-48 hours. After incubation, the lid of Calgary device was then washed and transferred to a challenge plate which containing polymer solution with two-fold dilution with total volume of 200 μ L. After incubated at room temperature for 2 hours, the lid was then again removed from the challenge plate and transferred to the recovery plates containing neutralizer. The recovery plate was sonicated for 30 $\pm$ 5 min to remove and disaggregate the biofilm. The suspensions were diluted with PBS and plated on agar plates to determine CFU. To determine the MBC values of FTP NPs, 20 μ L of the solutions in challenge plate was transferred into a fresh 96-well plate containing 180 μ L TSB and incubated at 37 °C for 24 h. The MBC values were determined by measuring OD at 650 nm wavelength.

The crystal violet (CV) stain assay was conducted following a published report with minor modification.¹⁹ Briefly, 150 μ L of the bacterial suspension in TSB (10^5 CFU/mL) was added into 96-well plate. After 24 hours incubation at 37 °C, the preformed biofilms were washed with DI water and followed by adding different concentrations of polymer solution with a total volume of 200 μ L in each well. After

2 hours treatment, the 96-well microplates were washed with DI water and stained with 100 μ L of 0.1 % CV solution at room temperature for 15 minutes. After staining, the microplates were then washed with DI water and added 200 μ L of 37 % acetic acid to dissolve CV. Biofilm biomass reduction was recorded by measuring the OD₅₅₀ with a TECAN microplate reader.

Minimum Bactericidal Concentrations (MBCs)

To determine the MBC values of FTP NPs, PMET and vancomycin. 20 μ L of the solutions in the challenge plate wells was transferred into a fresh 96-well plate containing 180 μ L MHB and incubated at 37 °C for 24 h. After incubation, bacteria were then 10-fold serial diluted in sterile PBS and spread on agar plates. The MBC was designated as the lowest concentration at which no bacterial colonies were formed on agar plates. The untreated bacteria suspension was employed as negative control, while vancomycin was used as positive control.

CLSM for Planktonic Bacteria and Biofilms

LIVE/DEAD BacLight™ bacterial viability kit was used to investigate the membrane permeability before and after PMET treatment. Green-fluorescing SYTO 9 can enter all cells, live or dead, whereas red fluorescing propidium iodide (PI) can only stain the DNA of died/dying bacteria cell with damaged cytoplasmic membranes.⁷⁷ Briefly, MRSA USA300 were grown into mid-log phase in MHB, washed twice with PBS and diluted into PBS to $10^5 - 10^6$ CFU/mL. The polymer solution was added into the bacterial suspension at 1 X MIC for 30 mins. The cell images were captured using the Zeiss LSM780 confocal laser scanning microscope

(CLSM; Carl Zeiss, Jena, Germany) with a 63× plan-apochromatic oil immersion objective lens (numerical aperture, 1.46).

Time-lapse images were captured to study the dynamics of biofilm dispersal effect of FTP NPs. The MRSA USA300 biofilm was established on a sterile chambered coverglass slide by gently adding diluted culture ($10^5 - 10^6$ CFU/mL) and incubating statically at 37 °C for 24-48 hours. After washing the glass slide using PBS, bacteria were stained with SYTO 9 and PI. FTP NPs at 128 µg/mL were then gently introduced into the cover slide and the cell images were captured at different time points by using the Zeiss LSM780 confocal laser scanning microscope (CLSM; Carl Zeiss, Jena, Germany) with a 63× plan-apochromatic oil immersion objective lens (numerical aperture, 1.46). SYTO 9 green fluorescent and PI were excited at 488 nm and 561 nm, respectively. The captured images were further processed with the Zen 2011 software (Carl Zeiss, Germany) and ImageJ software.

FESEM for Planktonic Bacteria and Biofilms

The morphology change of MRSA USA300 was performed as described previously.⁶⁴ MRSA USA300 were grown into mid-log phase in MHB and washed twice with PBS and then diluted into PBS to $10^7 - 10^8$ CFU/mL. The polymer solution was then added into the bacterial suspension at 1 X MIC for 30 mins. After incubation, bacteria were fixed by 2.5 % glutaraldehyde at 4 °C overnight followed by dehydration with ethanol of a gradient of concentrations (25 %, 30 %, 50 %, 70%, 90%, 100%) and air dried for 24 hours. The final sample was coated with platinum and imaged by FESEM (JEOL JSM 6701F).

For the biofilm images, preformed biofilm was established on the surface of pegs of MBEC plate as previously described. After challenging at desired polymer concentration, the peg lids were broken off using a pair of flame-sterilized pliers and rinsed in PBS. The pegs were then fixed in 2.5% glutaraldehyde at 4°C for 16 h, after which they were washed with PBS followed by dehydrated in 70% ethanol for 20 minutes and air dried for 24 h before specimen mounting and examination by FESEM (JEOL JSM 6701F).

***In Vivo* Murine Wound Biofilm Model of MRSA USA300 Infection**

Experiments were performed according to protocols approved by the institutional animal care and usage committee (IACUC) of the Nanyang Technological University (protocol approval number IACUC A18051). Male C57BL6 mice (Invivos, Singapore) aged 7-8 weeks were used in the *in vivo* studies. Mice (n = 5 per group) were anesthetized using isoflurane and an excision wound was created on the dorsal area using a 5 mm diameter biopsy punch. 2.5 µL MRSA USA300 in PBS suspension (5×10^5 CFU/mL) was dispensed to wound site using a 10 µL pipet and the wound were subsequently covered by Tegaderm (3M™) to prevent contamination. After 24-hour inoculation, Tegaderm film was removed and first dose of vancomycin, PMET, FTP NPs or PBS (negative control) were added to infected wounds at 4 mg/kg. Afterwards, mice were returned to their cages and rested for 4 hours. 2nd (3 mg/kg) and 3rd (3 mg/kg) treatments were subsequently applied with 4-hour intervals. 4 h after last treatment, mice were sacrificed using CO₂ and tissue samples were harvested using scalpel blade. Samples were further homogenized and plated on agar plates to determine CFU.

In Vivo Toxicology Evaluation

Experiments were performed according to protocols approved by animal ethics and welfare of Ningbo University (protocol approval No. AEWC-2018-07). 10 mg/kg of PMET or FTP NPs were injected intravenous (i.v.) into female balb/c mice (n = 5 mice per group). Mice without injection were used as control. Mice weight and condition were monitored daily. At 1 day and 7 days' post-injection, mice blood was collected from submandibular vein and blood biochemistry was analyzed using Blood Chemistry Analyzer (Pointcare V2, MNCHIP).

For LD₅₀ determination, ICR mice (female, 5 weeks) were randomly divided into four groups (six mice per group). Each of the mice received single intraperitoneal injection of FTP NPs at varied concentrations (*i.e.* 20 mg/kg, 30 mg/kg, 40 mg/kg and 50 mg/kg). Mice health condition was monitored over 7 days and LD₅₀ was estimated from the survival rate of treated groups. The LD₅₀ determined for FTP NPs is 40mg/kg.

Statistical analysis was performed using either student's t-test or one-way analysis of Anova with Dunnett's correction where appropriate. *P*-values <0.05 were considered statistically significant.

5.3 Results and Discussion

PMET was first synthesized by reacting linear PEI with dicyandiamide under acidic conditions (**Scheme 5.1a**).²³⁴

Scheme 5.1 (a) Synthesis of PMET by reacting linear PEI with dicyandiamide. (b) Preparation of FTP NPs.

¹H NMR and GPC data (**Figure 5.1**) confirmed the successful synthesis of PMET. The degree of biguanide substitution (75 % - 78 %) of PMET was calculated from the ratio of the areas of the proton peaks of biguanide (“c” and “d”: 3.0 - 4.0 ppm, **Figure 5.1a**) to the total areas of unsubstituted PEI peaks (“a” and “b”: 2.5 - 2.7 ppm) plus the biguanide peak areas (“c” and “d”: 3.0 - 4.0 ppm). The measured molecular weight of PMET was 7.9 kDa which was close to the calculated value of 8.0 kDa for a starting PEI with molecular weight of 3.7 kDa (**Figure 5.1b**) with a grafting ratio of 75%-78%.

Figure 5.1 (a) ^1H NMR of Linear PEI in DMSO- d_6 and PMET in D_2O . (b) GPC of linear PEI and PMET.

Introducing biguanide group into PEI significantly improved the solubility of PMET in a neutral (physiological) pH aqueous environment, making it favorable for biological applications (**Figure 5.2**).

Figure 5.2 Visual assessment of solubility of linear PEI (10 mg/mL) and PMET (10 mg/mL) in PBS.

Table 5.1 Solution appearance of mixtures of PMET and TA at different mass ratios.

Samples	Solution turbidity
PMET	clean
TA	clean
PMET:TA =1:2	turbid
PMET:TA =1:1	turbid
PMET:TA =2:1	turbid
PMET:TA=10:1	turbid

Figure 5.3 (a) Optical images of turbidity of TA, PMET and TP NPs. (b) TEM image of TP NPs. Scale bar=100 nm.

TA/PMET (TP) NPs was formed by mix the TA and PMET at different mass ratios (**Table 5.1** and **Figure 5.3a**). TEM showed that these TP NPs have diameters of around 96 nm (**Figure 5.3b**). DLS data showed that the TP NPs have hydrodynamic diameter (D_h) of 96.9 ± 1.3 nm (**Table 5.2**), in excellent agreement with the TEM data. TP NPs are positively charged with a zeta potential of $+43.1 \pm 1.7$ mV. However, TP NPs exhibited poor stability in PBS, precipitating from suspension overnight at room temperature.

Table 5.2 DLS and zeta potential of PMET, TA, TP NPs and FTP NPs

Samples	Size (nm)	ζ (mV)
PMET	1.0 ± 0.2	14.5 ± 0.7
TA	2.2 ± 0.7	-1.3 ± 0.5
TP NPs	96.9 ± 1.3	43.1 ± 1.7
FTP NPs	121.6 ± 3.5	33.6 ± 1.4

To improve the colloidal stability of the NP, I further PEGylated the TP complex by introducing the biocompatible surfactant Pluronic® **F**-127 (FTP NPs) (**Scheme 1b**). Firstly, the preparation and optimization of FTP NPs were studied by mixing different mass ratios of F-127, TA and PMET. Here, PMET was kept at a constant concentration of 10 mg/mL with a constant ratio (1:1) of TA and F-127. When the concentration of TA or F-127 was below 1 mg/mL, it was found that the fresh prepared FTP NPs can be easily form nanostructure ($D_h = 85.0 \pm 2.6$ nm) but again precipitated out at room temperature, which have similar phenomenon with that of TP NPs. Moreover, when the concentration of TA or F-127 was higher than 1 mg/mL, micro-aggregates with $D_h = 4.5 \pm 0.7$ μ m were observed. These results indicated that the concentrations of F-127 and/or TA plays essential role in the formation of stable FTP NPs, and the optimized concentrations of F-127, TA and PMET was 1 mg/mL, 1 mg/mL and 10 mg/mL, respectively. After the stable FTP NPs were prepared, they were washed three times using a 50 kDa membrane ultrafiltration unit under centrifugation (5,000 r.p.m. for 30 min at 4 °C). The upper compartment of the centrifugal filter unit contained FTP NPs and the lower compartment contained filtrate. I then measured ^1H NMR and UV-Vis absorption spectra of the filtrate (**Figure 5.4**) to test for the presence of any components (F-127, TA or PMET) in it. No sign of these three components was detected in the ^1H NMR and UV-Vis absorption spectra, indicating that all three components were fully incorporated into the FTP NPs.

Figure 5.4 (a) ^1H NMR and (b) UV-Vis absorption spectra of filtrate in lower compartment of centrifugal filter units. (c) and (d) UV-Vis absorption of free PMET and TA as standard with different concentrations.

DLS measurements of FTP NPs indicate $D_h = 121.6 \pm 3.5$ nm, larger than that of TP NPs at 96.9 ± 1.3 nm, which may be attributed to the hydrophilic PEG chains extending from the particle surface. FTP NPs are positively charged with a zeta potential of $+33.6 \pm 1.4$ mV (**Table 5.2**). The diameter of FTP NPs measured in TEM images was around 111 nm (**Figure 5.5a**), corroborating the D_h measured by DLS. Prolonged standing study of the colloidal stability of FTP NPs showed that the D_h and polydispersity index (PDI) remained almost unchanged over two weeks (**Figure**

5.5b). These results indicate the successful fabrication of stable and uniform FTP NPs.

Figure 5.5 Characterizations of FTP NPs. (a) Hydrodynamic diameter (D_h) of FTP NPs using DLS. Inset is the TEM image of FTP NPs with scale bar = 200 nm. (b) Stability of FTP NPs no significant change of D_h and PDI with passage of time. Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

MICs of neat PMET were measured against a panel of *S. aureus* and MRSA strains (including community-associated MRSA USA300 and hospital-associated MRSA BAA40 and KKH5). PMET showed excellent anti-*Staphylococcal* and anti-MRSA activity with MICs of 8-16 $\mu\text{g/mL}$ (**Table 5.3**). However, for Gram-negative bacteria PMET showed moderate killing activity with MICs of 32-64 $\mu\text{g/mL}$ against various strains (*i.e.* *E. coli* ATCC8739, *P. aeruginosa* PAO1, *K. pneumoniae* KPNR and *A. baumannii* AB-1). FTP NPs showed similar antibacterial activity to PMET against both Gram-positive and Gram-negative bacteria.

Table 5.3 Antimicrobial of PMET, FTP NPs and reference antibiotics against planktonic Gram-positive and Gram-negative bacteria.

	MICs: µg/mL							
	Gram-positive				Gram-negative			
	<i>S. aureus</i> (ATCC 29123)	Methicillin resistant <i>S. aureus</i> (MRSA BAA40)	Methicillin resistant <i>S. aureus</i> (MRSA USA300)	Methicillin resistant <i>S. aureus</i> (MRSA KKH5 ^a)	<i>E. coli</i> (ATCC 8739)	<i>P. aeruginosa</i> (PAO1)	<i>K. pneumoniae</i> (KPNR)	<i>A. baumannii</i> (AB-1)
PMET	16	8	16	8	32	32	64	64
FTP NPs	16	8	16	8	16/32	32	64	64
Vancomycin	1	1	0.5	0.5	128	>128	>128	>128
Colistin	128	>128	128	128	2	1	2	2

^aMRSA KKH5 provided by KK women's and children's hospital, Singapore

The ability of FTP NPs and PMET to disperse preformed biofilm of MRSA USA300 was evaluated using the MBEC^{TM} assay. Biofilm was established on MBEC^{TM} pegs and then the pegs were soaked in wells containing PMET or FTP NPs. Biofilms could be dispersed by PMET and FTP NPs solutions. The biofilm dispersal ability of FTP NPs is better than that of PMET (**Table 5.4**): at concentrations of 8, 16, and 32 µg/mL, FTP NPs showed log₁₀ reductions of 2.4, 2.6 and 3.5 respectively, while PMET had much lower (0.2, 0.4 and 1.5) log₁₀ reductions at the same concentrations. The cell count reduction of PMET plateaued above 32 µg/mL at about 3.4 log₁₀. In contrast, FTP NPs showed a continual reduction of cell counts in a dose-dependent manner and better efficacy than PMET at all concentrations. At the highest tested concentration, 512 µg/mL, there was almost complete bacterial removal (5.2 log₁₀ reduction) for FTP NPs compared to partial removal (3.2 log₁₀ reduction) for PMET.

Table 5.4 Log₁₀ reduction of MRSA USA300 biofilm cell counts treated by FTP NPs, PMET and vancomycin compared to untreated control.

Concentrations ($\mu\text{g/mL}$)	Samples		
	FTP NPs	PMET	Vancomycin
4	1.1	-0.1	0.1
8	2.4	0.2	0.9
16	2.6	0.4	0.7
32	3.5	1.5	0.9
64	3.6	3.4	0.8
128	4.0	3.5	1.1
256	4.2	3.2	1.1
512	5.2	3.2	1.4

To assess the agents' penetration into and action on biofilm matrix and cells, MRSA USA300 biofilm was formed on glass slides and incubated with rhodamine labelled FTP NPs and PMET at 16 $\mu\text{g/mL}$ for 30 mins. The penetration into and accumulation of FTP NPs and PMET in biofilm were examined by CLSM.

Figure 5.6 (a) Penetration and accumulation of rhodamine-labeled (red) FTP NPs (upper panel) and PMET (lower panel) into MRSA USA300 biofilm (Syto 9: green) at 16 $\mu\text{g/mL}$ for 30 min. (b) Fluorescence intensities of rhodamine-labeled (red) FTP NPs and PMET as a function of depth in the biofilm

(dashed vertical line represents the bottommost layer of biofilm). Scale bar is 20 μm .

As shown in **Figure 5.6**, FTP NPs showed more red fluorescence in the biofilm than did PMET after 30 mins treatment. Further, FTP NPs showed reduced green fluorescence (Syto 9: live bacteria) compared to PMET. Using ImageJ software to analyze the CLSM images, the penetration and accumulation of FTP NPs and PMET was quantified; the fluorescence intensity as a function of biofilm depth is shown in **Figure 5.6**. The FTP NPs penetrated with high concentration significantly deeper into the biofilm than did PMET. In a separate experiment, CLSM images of MRSA USA300 biofilms treated with PMET and FTP NPs (**Figure 5.7**) at the concentration of 16 $\mu\text{g/mL}$ for 2 h showed that FTP NPs have better biofilm removal ability than PMET. There is a clear difference in the biofilm thickness: 12.8 μm after PMET treatment *versus* 3.2 μm after FTP NPs treatment.

Figure 5.7 3D confocal microscopy images of MRSA USA300 biofilms treated with PMET (upper panel) and FTP NPs (lower panel) at 16 $\mu\text{g/mL}$ for 2 h. Live and dead bacterial cells were stained by Syto 9 (green) while only dead cells were stained by propidium iodide (red).

Using FESEM, the biofilm condition before and after treatment with FTP NPs was visualized. FESEM images (**Figure 5.8a**) showed a clear bacterial count reduction after a single dose (128 $\mu\text{g/mL}$) FTP NP treatment.

Figure 5.8 (a) FESEM images of MRSA USA300 biofilm before and after FTP NPs treatment (128 $\mu\text{g/mL}$). Scale bars are 1 μm . (b) Minimum bactericidal concentration (MBC) values of FTP NPs and PMET against dispersed planktonic bacteria in the MBEC^{TM} challenge wells, measured by CFU count (circles represent zero count). Untreated bacteria suspension was employed as negative control, while vancomycin was used as positive control. Data are presented as mean $\pm$ standard deviation and represent three independent experiments. (c) 3D confocal images of MRSA USA300 biofilms treated by FTP NPs at 128 $\mu\text{g/mL}$ with incubation time: 0 min, 30 min, and 120 min, showing the removal of MRSA USA300 biofilm.

Further, the ability of FTP NPs to kill bacteria dispersed from biofilm was examined. The bacteria were grown on MBEC^{TM} pegs to the biofilm state by submerging the pegs into a 96-well plate containing growth medium (for 24 h); the

pegs were then placed into another 96-well plate containing the FTP NPs solution for 2 h. the viability of bacteria dispersed from the biofilm on the pegs was then examined; I transferred 20 μ L of the dislodged bacteria suspension (containing the polymers and the dispersed-from-biofilm cells) into a fresh 96-well plate containing 180 μ L growth medium and then incubated the bacteria with medium for 24 h before counting the colony forming unit (CFU) to determine the minimum bactericidal concentration (MBC) value of FTP NPs against bacteria dispersed from biofilm. It was found that the FTP NPs have “intrinsic biofilm bactericidal activity” – the ability to kill bacteria *in situ* within the biofilm as well as after release from the biofilm through the agent’s biofilm dispersal effect -- and this is significantly better than many previously reported AMPs or polymers that do not do both well.^{10, 56, 232} The FTP NPs have “dispersed from biofilm” bacterial MBC of 128 μ g/mL which is slightly better than PMET, which has MBC of 256 μ g/mL (**Figure 5.8b**).

Time-lapse CLSM confirmed the biofilm reduction effect of FTP NPs via removal of the biomass and killing of biofilm bacteria (**Figure 5.8c**). MRSA USA300 biofilms were pre-formed on the surface of glass slides and then FTP NPs (128 μ g/mL) solution was dispensed on the biofilm before staining by LIVE/DEAD^{TM} dye. CLSM images (**Figure 5.8c**) showed that FTP NPs can disperse the biofilm effectively in a very short period of time (30 min) with significant reduction of live bacteria (green fluorescence in **Figure 5.8c**) and dispersal of killed cells from the matrix (little red fluorescence from residual dead cells compared with the initial green fluorescence). After 120 min, few bacteria remained in the biofilm.

I also tested the cytotoxicity of FTP NPs and PMET against two mammalian cells. The results (**Figure 5.9a**) show that FTP NPs have good biocompatibility with 70% inhibitory concentration (IC_{70}) $>1024 \mu\text{g/mL}$ against 3T3 fibroblasts, a value much superior to the $IC_{70} = 239.8 \mu\text{g/mL}$ of PMET. For the human dermal fibroblasts (HDF), FTP NPs also showed much higher IC_{70} ($>1024 \mu\text{g/mL}$) than PMET ($125.6 \mu\text{g/mL}$) (**Figure 5.9b**). The results demonstrate that FTP NPs have better *in vitro* biocompatibility than PMET toward the eukaryotic cells tested, which is possibly due to hydrophilic PEG chain of F-127.

Figure 5.9 *In vitro* biocompatibility of PMET and FTP NPs towards mammalian cells. (a) mouse embryonic fibroblast 3T3 cells. (b) HDF cells. Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

Figure 5.10 (a) MRSA USA300 membrane potential depolarization assessed by DiSC3(5) fluorescence after FTP NPs treatment. (b) FESEM images of MRSA USA300 (i) before and (ii) after FTP NPs treatment at MIC (16 μg/mL). Scale bar is 1 μm. (c) CLSM images of MRSA USA300 bacteria of (upper panel) untreated control and (lower panel) FTP NPs treatment at MIC (16 μg/mL). Live and dead bacterial cells were stained by Syto 9 (green) while only dead cells were stained by propidium iodide (red). Scale bar is 10 μm.

FTP NPs and PMET showed both biofilm removal and biofilm bacterial killing ability as mentioned above. To study the mechanism of action of FTP NPs and PMET against MRSA USA300, we used the cytoplasmic membrane depolarization assay with DiSC3(5) dye, FESEM and CLSM. Treatment with FTP NPs resulted in a rapid increase of the DiSC3(5) fluorescence intensity, indicating its effect in depolarizing the cytoplasmic membrane (**Figure 5.10a**). Treatment with PMET also resulted in an increase of the DiSC3(5) fluorescence intensity (**Figure 5.11a**). FESEM images (**Figure 5.10b**) showed morphology changes in FTP NPs-treated ($1 \times \text{MIC}$: $16 \mu\text{g/mL}$) bacteria, which had wrinkled and deformed surfaces compared with the smooth and intact surfaces of untreated bacteria. PMET treated bacteria ($1 \times \text{MIC}$: $16 \mu\text{g/mL}$) also showed surface deformations in FESEM imagery (**Figure 5.11b**). CLSM was then utilized to observe MRSA USA300 bacteria stained with Syto 9 (green) and propidium iodide (red). For the untreated control (**Figure 5.10c**), most of cells were green and not stained by PI, indicating intact bacterial cell membrane. In contrast, both FTP NPs and PMET-treated ($1 \times \text{MIC}$: $16 \mu\text{g/mL}$) bacterial cells (**Figure 5.10c and 5.11c**) were stained by red-fluorescent PI dye, indicating dead bacteria with disrupted cell membrane. The agglomeration of bacteria and formation of clusters of MRSA was observed after treated by FTP NPs and PMET. The clustering may due to a response to environment stress caused by antimicrobial agents such as FTP NPs and PMET, which is similar to some previous studies.^{9, 237}

Figure 5.11 (a) MRSA USA300 membrane potential depolarization assessed by DiSC3(5) fluorescence after FTP NPs treatment. (b) FESEM images of MRSA USA300 before and after PMET treatment at MIC (16 µg/mL). Scale bar is 1 µm. (c) CLSM images of MRSA USA300 bacteria with (upper panel) no treatment (control) and (lower panel) PMET treatment at MIC (16 µg/mL). Live and dead bacterial cells were stained by Syto 9 (green) while only dead cells were stained by propidium iodide (red). Scale bar is 10 µm.

A liposome model of bacterial plasma membrane was employed in a calcein dye leakage assay to obtain a quantitative measure of membrane permeabilization caused by FTP NPs and PMET. Calcein was loaded into phosphatidylglycerol (PG)/cardiolipin (CL) liposomes (PG:CL = 3:1), which were used to mimic Gram-positive bacterial membrane. FTP NPs induced ~50% dye leakage from PG/CL liposome at 16 $\mu\text{g/mL}$ (1 x MIC); at this wt/vol concentration, PMET induced around 30% leakage (**Figure 5.12**). These results corroborate the interpretation that both FTP NPs and PMET kill bacteria by a mechanism involving membrane permeabilizations.

Figure 5.12 Calcein dye leakage caused by addition of FTP NPs and PMET at 16 $\mu\text{g/mL}$ (1 x MIC). Liposome composition: PG/CL (3:1, w/w; membrane mimic of Gram-positive MRSA USA300) vesicles. Triton X-100 was employed as positive control to cause 100% dye leakage.

The time-kill kinetics of FTP NPs and PMET against planktonic MRSA USA300 was investigated by CFU assay. As shown in **Figure 5.13**, at 1 x MIC concentration, both FTP NPs and PMET achieved 5.6 log₁₀ reduction of MRSA USA300 in 4 h. Vancomycin showed much poorer killing efficacy (~1.6 log₁₀ reduction) after 4 h treatment at 1 x MIC. At 2 x MIC and 4 x MIC, FTP NPs and PMET both achieved 5.6 log₁₀ reduction of the bacteria cell counts within 60 mins at 2 x MIC and within 5 mins at 4 x MIC. Vancomycin required the full 4 h test period to achieve 5.6 log₁₀ reduction at both 2 x MIC and 4 x MIC. Against MRSA USA300, FTP NPs and PMET exhibit much faster kill kinetics than vancomycin.

Figure 5.13 Killing kinetics of planktonic MRSA USA300 at different concentrations: (a) 1 x, (b) 2 x and (c) 4 x MIC of FTP NPs and PMET. Untreated bacteria was employed as negative control, while vancomycin was used as positive control. Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

The interaction between PMET and TA was studied by investigating the mixture's optical property (turbidity) and isothermal titration calorimetry (ITC) behavior. Turbid suspension was formed at all tested PMET to TA mass ratios (**Figure 5.3** and **Table 5.1**). The ITC curve (**Figure 5.14a**) showed a negative enthalpy change ($\Delta H =$

-57.3 $\pm$ 3.78 kcal/mol) and a positive entropy change (- Δ S= 48.0 kcal/mol), indicating the interaction between TA and PMET was enthalpically driven with unfavorable entropic change; this thermodynamic behavior and the known structures of the two components implies they bind primarily through hydrogen bonding. F-127 has previously been shown to interact with TA by hydrogen bonding.^{236, 238} Fourier transform infrared spectrum (FTIR) was utilized to study the interaction between TA and F-127 (**Figure 5.14b**). The results showed that the C=O stretching vibration in TA shifted from 1713 cm⁻¹ to 1731 cm⁻¹ in the F-127/TA mixture. Also, compared to TA, the F-127/TA mixture showed a red shift in the -OH peak (from 3463 cm⁻¹ in TA to 3605 cm⁻¹ in the mixture), indicating the formation of hydrogen-bonding between TA and F-127. In summary, FTP NPs can be formed with TA as building block to interact with both F-127 and PMET (**Scheme 5.1b**).

Figure 5.14 (a) ITC data: titration of TA (1.47 mM) into PMET (147 μ M), indicating the interaction between TA and PMET was enthalpically driven with

unfavorable entropic change. (b) FTIR spectra of TA, F-127 and F-127/TA mixture (1:1, w/w).

A murine excisional wound model was employed to examine the *in vivo* antibiofilm efficacy of FTP NPs. MRSA USA300 biofilms were first established by inoculation of 10^5 CFU/mL bacteria suspension onto excision wound sites (**Figure 5.15a**). Starting at 24 h post infection, a topical treatment with either 10 mg/kg vancomycin, FTP NPs, or PBS vehicle (control) was applied onto the infected wound site (three doses at 4-hour intervals: *i.e.* 4 mg/kg at 24 h post infection, followed by 3 mg/kg 4 h later, and another 3 mg/kg 4 h after the 2nd treatment). As shown in **Figure 5.15a**, FTP NPs suppress MRSA USA300 biofilm bacteria with a $\log_{10}$ reduction of 1.8, which is significantly ($p \leq 0.01$) better than vancomycin (with a $\log_{10}$ reduction of around 0.8), indicating the superior antibiofilm efficacy of FTP NPs. Furthermore, FTP NPs showed slightly higher antibiofilm efficacy than that of PMET alone (1.2 $\log_{10}$ reduction), which might be attributed to the nonspecific interaction with the biomolecules in biofluid of wound.²³⁹ Nevertheless, FTP NPs still showed significantly ($p \leq 0.05$) better in the reduction of biofilm cell counts than that of PMET.

Figure 5.15 (a) Illustration of murine wound model and *in vivo* antibiofilm activity. Log₁₀ CFU per wound from PBS alone (control), vancomycin (10 mg/kg), PMET (10 mg/kg) and FTP NPs (10 mg/kg). ns: no significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ by two-tailed Student's *t*-test. (b) Mice weight monitoring for 7 days post intravenous injection of FTP NPs at 10 mg/kg. The average weight was plotted *versus* time, with error bars representing the sample standard deviation within the experimental group at each day. Blood biochemistry analysis at 1 day and 7 days post intravenous injection of FTP NPs at 10 mg/kg. Blood biochemical parameters from each mouse are plotted as individual points and error bars represent the sample standard deviation within an experimental group. *P* values were calculated using one-way ANOVA analysis.

To determine the *in vivo* biocompatibility of FTP NPs, the material was intravenously injected into mice at 10 mg/kg followed by weight measurement and blood biochemistry analysis. The results (**Figure 5.15b**) showed no obvious weight loss and no change in behavior of mice receiving this substantial systemic dose of FTP NPs. Further, the blood biochemical analysis results demonstrated that no nephro- or hepato-toxicity was induced by intravenous injection of FTP NPs, as indicated by the negligible change of biomarkers (*i.e.* alanine transaminase (ALT), aspartate transferase (AST), blood urea nitrogen (BUN), and creatinine (CRE)) for treated groups 1-day and 7-days post injection. The LD₅₀ value (single lethal dose resulting in 50% mortality) was also determined via intraperitoneal injection into mice, and the results showed that FTP NPs have LD₅₀ at 40 mg/kg. Histological examination revealed that the tissues around wound after FTP NPs treatment have normal skin structure and less inflammatory cell infiltration as compared to the skin tissues of MRSA USA300 infected group (**Appendix Figure A6**). Overall, these data support that our biguanide-based FTP NPs can effectively suppress MRSA USA 300 biofilm in a murine model with negligible acute toxicity.

It is expected that the biguanide group of metformin to provide multiple hydrogen-bonding donor sites, making it versatile as a building block for supramolecular structure and/or NP formation.²³³ Further, it was showed that PMET can form NPs with the natural polyphenol TA which can serve as building block with strong affinity to multiple substances.²⁴⁰ The colloidal stability of TP NPs was improved by adding the third component F-127, which introduces PEG into the NPs (**Scheme 5.1b**), the resultant FTP NPs have good stability with almost unchanged

particle size over two weeks (**Figure 5.1b**). Further, these FTP NPs, compared with neat PMET alone, have better reduction (by $> 2 \log_{10}$) of biofilm cell counts at low concentrations (8-32 $\mu\text{g/mL}$) in MBEC^{TM} assay (**Table 5.4**).

3D CLSM images (**Figure 5.6**) demonstrated that FTP NPs, with their antifouling PEG content, have better biofilm penetration and accumulation than PMET and better biofilm removal ability (**Figure 5.7**) than PMET alone at 16 $\mu\text{g/mL}$ (with residual biofilm thickness of 12.8 μm after PMET treatment as opposed to 3.2 μm thickness after FTP NPs treatment). FTP NPs also showed almost complete biofilm eradication ($5.2 \log_{10}$ reduction) at the concentration 512 $\mu\text{g/mL}$. Further, FESEM and time lapse CLSM images confirmed a clear reduction of bacterial counts within biofilm after a single dose (128 $\mu\text{g/mL}$) treatment of FTP NPs. All the results demonstrated that the FTP NPs have better antibiofilm efficacy than PMET alone. The dispersed planktonic bacterial killing ability of FTP NPs was also determined and the results showed that FTP NPs can kill bacteria dispersed from biofilm with MBC of 128 $\mu\text{g/mL}$. PMET also kills bacteria dispersed from biofilm with MBC of 256 $\mu\text{g/mL}$.

FTP NPs can disperse biofilm bacteria and also kill the dispersed bacteria. Various assays, including DiSC3(5), CLSM, FESEM, calcein dye leakage and killing kinetics, was utilized to study the antibiofilm mechanism of FTP NPs and the results strongly suggest that the bacterial kill mechanism of FTP NPs involves membrane permeabilization. FTP NPs are superior to dextran-*block*-poly(AMPTMA-*co*-BMA) that only shows biofilm dispersal effect -- “nanoscale bacterial debridement” -- without bactericidal effects.²³² Agents that disperse biofilm

without killing the bacteria entail the risk that the dispersed bacteria can disseminate into and infect other parts of the body, unless an antibacterial agent is co-administered. It is hypothesized that the improved efficacy of FTP NPs may be attributed to the hydrophilic PEG chain of F-127 dangling at the surface of the NPs (**Scheme 5.1b**). This antifouling chain could facilitate the NPs penetration into the biofilm which was confirmed by confocal microscopy, so that the NPs can kill bacteria throughout the depth of the biofilm matrix. FTP NPs showed good ability to eradicate preformed biofilm by penetrating into the biofilm matrix and then killing the bacteria. The bacterial kill mechanism appears to involve membrane permeabilization; attachment of the NPs is presumably promoted by electrostatic interaction between the positively charged NPs and anionic bacterial envelope. FTP NPs are also able to kill live bacteria that detach from the biofilm matrix makes them a superior antibiofilm agent.

5.4 Conclusions

In this chapter, it has demonstrated for the first time that PMET has good anti-MRSA ability. TA, a polyphenol and F-127, a poloxamer, were introduced to assist the formation of FTP NPs. With good biofilm penetration and dispersion ability and intrinsic biofilm bacterial killing activity, FTP NPs can remove MRSA USA300 biofilm more effectively than neat PMET as shown by the *in vitro* MBEC^{TM} assay. In *In vivo* murine wound infection testing, FTP NPs exhibit significantly ($p \leq 0.01$) higher reduction ($1.8 \log_{10}$) in biofilm bacteria than the antibiotic vancomycin ($0.8 \log_{10}$ reduction). In *in vitro* biocompatibility tests, FTP NPs are less cytotoxic than PMET towards representative mammalian cells (3T3 and HDF). FTP NPs cause no

acute *in vivo* toxicity to mice at 10 mg/kg systemic dose, with negligible loss of body weight and change of blood biomarkers. It is expected that these FTP NPs can provide an alternative strategy to treat MRSA biofilm associated wound infections and other MRSA related infections.

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Chapter 6 Conclusions and Perspective

6.1 Conclusions

In this thesis, I first developed a new series of cationic dextran-*block*-poly(AMPTMA) copolymers by applying ATRP. The optimized cationic DA100 plays an important role in the antimicrobial activity against some clinic strains of Gram-positive *S. aureus*, including MRSA, with the MICs of 8-16 $\mu\text{g/mL}$; and also shows biofilm inhibition against Gram-negative *E. coli*. The highly selectivity is mainly attributed to the different binding interaction of the cationic polymers with the surface of Gram-positive and Gram-negative bacteria. Further, DA100 shows less cytotoxicity than A100 towards mammalian cells and non-haemolytic. However, both A100 and DA100 are incapable to remove the preformed biofilms of both Gram-negative and Gram-positive bacteria, which highly possibly due to the poor penetration ability into biofilm.

Therefore, a novel antibiofilm cationic copolymeric NP has been synthesized to study both the antibacterial and antibiofilm ability by simply introduction of small amount of hydrophobic BMA into DA100. The DA95B5 NP is then self-assembled from dextran-*block*-poly(AMPTMA)-*co*-(BMA). Interestingly, DA95B5 don't have any antibacterial effect but excellent preformed biofilm removal ability with a mechanism called "nanoscale bacterial debridement". The *in vitro* results show that DA95B5 have the antibiofilm ability towards several multi-drug resistant and clinically relevant strains, with the efficacy much higher and/or similar to the conventional standard antibiotics. *In vivo* data (using a murine excisional wound model) also show that DA95B5 can effectively disperse MRSA biofilm with $\log_{10}$

reduction up to 3.6, which is significantly better than the efficacy of the last resort antibiotic vancomycin ($1.7 \log_{10}$ reduction). Further, both the *in vitro* and *in vivo* data show these NPs have good biocompatibility with low hemolysis and cytotoxicity. Overall, this novel biofilm removal approach provides exciting opportunities for treatment of MDR biofilm infections and which further may have widespread applications.

Lastly, I introduced FTP NPs with the good penetration ability and intrinsic biofilm bacterial killing activity. FTP NPs can remove MRSA USA300 biofilm more effectively than PMET by MBEC assay *in vitro*. *In vivo* murine wound infection model also demonstrated that FTP NPs have antibiofilm efficacy much higher compared to antibiotic vancomycin ($0.8 \log_{10}$ reduction). Furthermore, *in vitro* biocompatibility data shows FTP NPs has less cytotoxicity towards different mammalian cells (including 3T3 and HDF); Moreover, the *in vivo* data also shows FTP NPs didn't cause acute toxicity to mice with negligible loss of body weight and change of blood biomarkers at dose of 10 mg/kg. It is expected that these FTP NPs can provide an alternative method to treat MRSA biofilm associated wound infections and other MRSA related infections.

6.2 Future Directions

Based on the understanding of structure of biofilm and conclusions of this thesis, many strategies would be developed to treat biofilm-associated infections by similar nanoplateforms to target EPS and microorganisms inside the biofilm. As for potential antibiofilm nano-agents for clinical applications, there are few main components or functions should be required in order to (1) have good biofilm penetration ability; (2)

target the bacteria inside biofilm to either kill the cells or weaken the interaction between cells and EPS; (3) minimize the toxicity effect on host cells. For instance, antifouling compounds such as polysaccharides, PEG, zwitterionic polymers could assist in the penetration and diffusion of nano-agents into biofilm, and they are also typically non-toxic to mammalian cells. Further, targeting agents (such as cationic polymers and/or sugar molecules) should be embedded in the system to selectively bind to bacterial cells as Chapter 4 described or to kill the bacteria inside biofilm as Chapter 5 described. Moreover, the laboratory research should be more focus on the *in vivo* efficacy and toxicity study to translate the potential agents to practical applications.

6.2.1 Smart System to Release Antimicrobial Agents

Comparing to the common delivery systems, smart formulations can enhance the effectiveness of antibiofilm activity by various triggers including pH,^{29, 30} temperature,²⁴¹ enzyme,¹⁸ electro- and magnetic- filed,¹¹⁶ and lights.²⁰ These systems can provide us much wider opportunities when facing different clinical situations. Besides, the combination of two or two more stimuli in one system will become more effective to remove biofilms. For example, many attentions have been attracted by the photodynamic therapy against biofilm infections.²³⁶ Hence, a potent antibiofilm nano-agent could be developed by combination of releasing antibacterial agents encapsulated in temperature-responsive materials while applying photothermal therapy. It is also expected that the high temperate generated by photothermal agent could promote the dispersal of biofilm but no harm to normal tissues, while the

releasing of antibacterial agents upon temperature change would eliminate the dispersed bacterial cells to treat the infections eventually.

6.2.2 New Nanotechnology for Antibiofilm

The hybrid of organic and inorganic materials may provide the chance to enhance the antibiofilm efficacy.¹¹⁹ With the low stability and penetration ability as well as high toxicity of current nanotechnology, the highly active inorganic nanomaterials still remained as a concern in aspect of *in vivo* biocompatibility. On the other hand, the low and moderate antibiofilm efficacy of organic nanomaterials usually non-toxic to mammalian cells. Therefore, a combination of these two kinds of materials would provide us the chance to treat biofilm infections. A promising example would be the nanozyme, which usually made from inorganic materials such as metal and silica. It also can be easily modified by non-toxic polymers to provide multifunction, enhance their stability and penetration ability. Hybrids of polydopamine and metal NPs (iron oxide NPs,²⁴² Au NPs,²⁴² silica NPs²⁴³) have been already studied as antibiofilm agents with good stability as well as antibacterial ability by production of ROS. Thus, a rational design by selecting the different organic and inorganic materials would provide many possibilities to develop novel systems with good antibiofilm activity as well as good biocompatibility both *in vitro* and *in vivo* in future.

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Appendix

Figure A1 ^1H NMR spectra of (a) DA95B5, (b) DA90B10, and (c) DA80B20. (d) GPC curves of DA95B5, DA90B10, and DA80B20.

Table A1 MICs of DAB copolymers series against bacterial strains.

Samples	MICs: $\mu\text{g/mL}$	
	<i>S. aureus</i> (MRSA BAA40)	<i>E. coli</i> (ATCC 8739)
DA95B5	512	512
DA90B10	>512	>512
DA80B20	>512	>512

Figure A2 *In vitro* biocompatibility of DA95B5, DA90B10, and DA80B20 towards 3T3 cells at 100 $\mu\text{g/mL}$. Data are presented as mean $\pm$ standard deviation and represent three independent experiments.

Figure A3 Penetration profile of DA95B5 into Gram-negative *E. coli* ATCC8739 at different time points. The x-axis is the depth of penetration of biofilms, where 0 μm represents the top layer of biofilm and $\sim 8.4 \mu\text{m}$ (represented by dashed vertical line) the bottommost layer of biofilm. The y-axis is intensity of red channels.

Figure A4 Live/Dead assay showing the cell viability of 3T3 cells. (a) Untreated control, (b) DA95B5 (100 $\mu\text{g/mL}$) and (c) DA95B5 (200 $\mu\text{g/mL}$). Cells with intact cell membranes were showing green fluorescence stained by calcein-AM; Cells with compromised membranes exhibit red-fluorescence from the live-cell-impermeant nucleic acid stain EthD1.

Figure A5. *In vivo* antibiofilm activity. Log₁₀ CFU per wound from PBS alone (control), FTP NPs with single dosage of 10 mg/kg, two treatments, and three treatments with total dosage of 10 mg/kg.

Figure A6. Histological images of tissues around wound after FTP NPs treatment. (a) MRSA USA300 infected tissues without treatment, (b) FTP NPs treated tissues (c) uninfected normal tissues. Scale bar=50 μ m.