Antimicrobial Polymers as Adjuvants/Synergists of Antibiotics to Combat Multidrug-Resistant Bacteria

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Thesis submission for the degree of Master of Philosophy

Thesis Title and Abstract | Declarations | Inclusion of Publications Statement | Corrected Thesis and Responses

Thesis Title

Antimicrobial Polymers as Adjuvants/Synergists of Antibiotics to Combat Multidrug-Resistant Bacteria

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The emergence of multidrug-resistant (MDR) bacteria due to the overuse and misuse of antibiotics in the medical and agricultural sectors has now become a critical global healthcare issue. Antimicrobial peptides (AMPs) and synthetic mimics thereof have shown promise in combating MDR bacteria effectively, mainly because of their mechanism of action that disrupts bacteria cell membrane, consequently hindering resistance development in bacteria. However, these antimicrobials also exhibit toxicity to healthy mammalian cells at high dosage. To overcome this toxicity issue, the application of combination therapy alongside traditional antibiotics could enable the administration of these membrane-disrupting antimicrobials at lower dosage. Herein, this thesis investigates the synergetic effects of new tri-systems for combination therapy against Gram-negative bacteria which contain: i) an AMP (colistin methanesulfonate); ii) an antimicrobial polymer as AMP mimic and; iii) commercial antibiotics. It was found that colistin and the antimicrobial polymer could combine synergistically with any of the three antibiotics, doxycycline, rifampicin, and azithromycin, against wild type and MDR strains of *Pseudomonas aeruginosa*. Crucially, given the lower dosage of antimicrobial polymer used in these combination systems, the therapeutic index (also known as selectivity index), which is an indicator of an antimicrobial system to preferentially target bacteria over mammalian cells, is higher than the standalone agents. Furthermore, in this thesis, other selected antimicrobial polymers that are active toward mycobacteria instead of Gram-negative were also investigated as potential adjuvants or synergists to potentiate the antimicrobial activity of antibiotics against *Mycobacterium smegmatis* via a two-component system. Among the different families of antibiotics screened, it was found that these polymers only act as adjuvants of aminoglycosides. Overall, this thesis yields valuable new insights on combination therapy that will be useful toward combating MDR bacteria in clinical settings.

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Publication Details #1

Full Title:	Two Plus One: Combination Therapy Tri-Systems Involving Two Membrane Disrupting Antimicrobial Macromolecules and Antibiotics
Authors:	Zeyu Shao, Erna Wulandari, Ruby C. Y. Lin, Jiangtao Xu, Kang Liang, and Edgar H. H. Wong
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The Candidate's Contribution to the Work:	Most of the experiments designing and conducting; data obtaining and analysis; manuscript writing
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Antimicrobial Polymers as Adjuvants/Synergists of Antibiotics to Combat Multidrug-Resistant Bacteria

Zeyu Shao

A thesis in fulfilment of the requirements for the degree of

Master of Philosophy (MPhil)

School of Chemical Engineering

Faculty of Engineering

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Abstract

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the antimicrobial activity of antibiotics against *Mycobacterium smegmatis* via a two-component system. Among the different families of antibiotics screened, it was found that these polymers only act as adjuvants of aminoglycosides. Overall, this thesis yields valuable new insights on combination therapy that will be useful toward combating MDR bacteria in clinical settings.

List of Publications

Zeyu Shao, Erna Wulandari, Ruby C. Y. Lin, Jiangtao Xu, Kang Liang, and Edgar H. H. Wong "Two Plus One: Combination Therapy Tri-Systems Involving Two Membrane Disrupting Antimicrobial Macromolecules and Antibiotics" *ACS Infect. Dis.*, submitted, contributed to this thesis, and described in Chapter 3

Table of Contents

List of Figures

List of Tables

List of Abbreviations

A	azithromycin dihydrate
A. baumannii	*Acinetobacter baumannii*
AIDs	acquired immune deficiency syndrome
AMPs	antimicrobial peptides
C	colistin sodium methanesulfonate
CAP	the tri-system with colistin, azithromycin, and polymer **P**
CDP	the tri-system with colistin, doxycycline, and polymer **P**
CFU	colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
CLSM	confocal laser scanning microscopy
CRP	the tri-system with colistin, rifampicin, and polymer **P**
D	doxycycline hydrochloride
DMEM	Dulbecco's modified eagle medium
DP	degree of polymerization
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EMB	ethambutol

FICI	fraction inhibitory concentration index
GSH	glutathione
HIV	human immunodeficiency virus
IC_{50}	half maximal inhibitory concentration
INH	isoniazid
M. abscessus	*Mycobacterium abscessus*
MBC	minimum bactericidal concentration
MDR	multidrug-resistant
MEFs	murine embryonic fibroblasts
MHB	Mueller Hinton broth
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
M. smegmatis	*Mycobacterium smegmatis*
M. tuberculosis	*Mycobacterium tuberculosis*
NAC	*N*-acetyl cysteine
NI	no interaction
P	a statistical ternary antimicrobial copolymer
P2	a linear homopolymer containing quaternary ammonium groups with 100 DP
P2-40	a linear homopolymer containing quaternary ammonium groups with 40 DP

P2b-40	a linear random copolymer with quaternary ammonium and hydrophobic phenyl groups with 40 DP
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	phosphate buffered saline
pET_20	a guanidium-functionalized polycarbonate
PET-RAFT	photo-induced electron transfer-reversible addition fragmentation chain transfer polymerization
P. hauseri	*Proteus hauseri*
RAFT	reversible addition-fragmentation chain transfer polymerization
R	rifampicin
S. aureus	*Staphylococcus aureus*
SEM	scanning electron microscope
TI	therapeutic index
Tween-80	polyethylene glycol sorbitan monooleate

Chapter 1

Introduction

The prolonged misuse of antibiotics in therapeutics and animal husbandry has led to the rise of multidrug-resistant (MDR) bacteria.[1] According to the Australian Department of Health,[2] antimicrobial resistance is currently a threat to global public health that requires urgent action, and is reported in Australia to be the 'Greatest threat to human health'[3] because of the 'Rise of the superbugs'.[4] By 2050, it is anticipated that drug-resistant infections could cause 10 million deaths worldwide, while costing the global economy up to \$100 trillion if no considerable measures are available.[5] Given that antibiotic resistance is developing faster than the introduction of new antimicrobial agents, there exists an urgent need for developing new and effective strategies to overcome MDR bacteria.

A promising approach to combat this global health challenge entails the use of synthetic cationic antimicrobial polymers,[6-14] which are essentially mimics of naturally occurring antimicrobial peptides (AMPs).[15-19] This class of compounds have been shown to minimize resistance development in bacteria mainly because of their ability to exert antimicrobial activity via the well-known physical membrane disruption mechanism.[20] While great progress has been made over the years in terms of designing antimicrobial polymers that exhibit potent activity towards MDR strains, however, most of these polymers still suffer from having low to moderate biocompatibility,[21, 22] as characterized by their narrow therapeutic index (TI) value. The TI is a reliable quantitative measurement that estimates the safety of an antimicrobial agent and its selectivity for bacteria over mammalian cells, and can be defined as the ratio of its IC_{50} (i.e., the half-

maximal concentration that reduces the viability of a mammalian cell by half) to MIC

(i.e., the minimum inhibitory concentration that inhibits bacteria growth) (**Equation 1.1**).

$$TI = IC_{50} / MIC$$

Equation 1.1. The equation of therapeutic index

To overcome the issue of toxicity in antimicrobial polymers, the use of combination

therapy such as the one involving the coadministration of antibiotics may prove to be an

effective avenue.[23-28] Specifically, if a particular combination demonstrates synergistic or

adjuvant effect, lower doses are required to achieve the same antimicrobial efficacy as

the individual compounds (hence lower MIC), which would then translate to higher TI

assuming that mammalian cells are unaffected by the overall potentiation in bioactivity

of the combination. In other words, the synergy or adjuvant action of an antimicrobial

combination is strictly confined to bacteria cells. Another advantage of combination

therapy involving antimicrobial polymers is the potential to revive the susceptibility of

'resisted' antibiotics in MDR bacteria (i.e., reversing resistance), thereby ensuring that

current antibiotics can still maintain their use.

In the following Chapter 2, a brief overview of the literature that describe the use of

antimicrobial polymers in combination therapy with antibiotics is described.

In Chapter 3, the development of new combination therapy tri-systems that include the

coadministration of two different membrane-disrupting type antimicrobial agents –

synthetic antimicrobial polymer **P** and the AMP colistin methanesulfonate **C** – in

conjunction with an antibiotic (doxycycline **D**, rifampicin **R** or azithromycin **A**) is described.

In Chapter 4, several antimicrobial polymers that are known to target mycobacteria (e.g., *Mycobacterium smegmatis*) were investigated for their ability to participate in combination therapy with different classes of antibiotics via a two-component system.

Finally in Chapter 5, the conclusions drawn from this thesis and the future outlook of this research are presented here.

Chapter 2

Literature Review

2.1 Antimicrobial Peptides

The antimicrobial peptides (AMPs) were also known as host defense peptides which were polycationic peptides with 7-100 amino acids. AMPs existed in the immune system of all eukaryotes and prokaryotes to assist the survival of the fittest in natural selection during the competition with other organisms.[20, 29] Thus, through the unremitting evolution over millions of years for surviving, AMPs became natural, highly efficient broad-spectrum antibiotics against wild pathogens even MDR strains because of their small size, hydrophobic structure and the cationic residue which distributed on its highly ordered amino acid sequences.[30] Till now, more than 3000 AMPs were reported, including the synthetical and natural products which could be found in several databases, such as CAMP$_{R3}$[31], LAMP[32], APD3[33], and ANTIMIC[34]. The generally accepted antimicrobial mechanism of AMPs is via the disruption of membrane this can be driven from various modes of actions, such as the electrostatic interaction between the cationic residue of AMPs and negatively charged cell membrane, the specific interaction with membrane protein, the inhibition of cell wall or membrane protein synthesis, which also might be the root of its cytotoxicity (the interaction between eukaryotes membrane and AMPs) as shown in **Figure 2.1**.[20, 35] It was noteworthy that the precise acting mechanism of AMPs still needed to be further elucidated, for instance, at the preliminary stage of membrane disruption, also known as the pore formation stage, several different permeabilization mechanisms of cationic AMPs on the cytoplasmic outer membrane were proposed, including 'Carpet', 'Barrel-Stave', and 'Toroidal' mechanism as shown in **Figure 2.2**.[36]
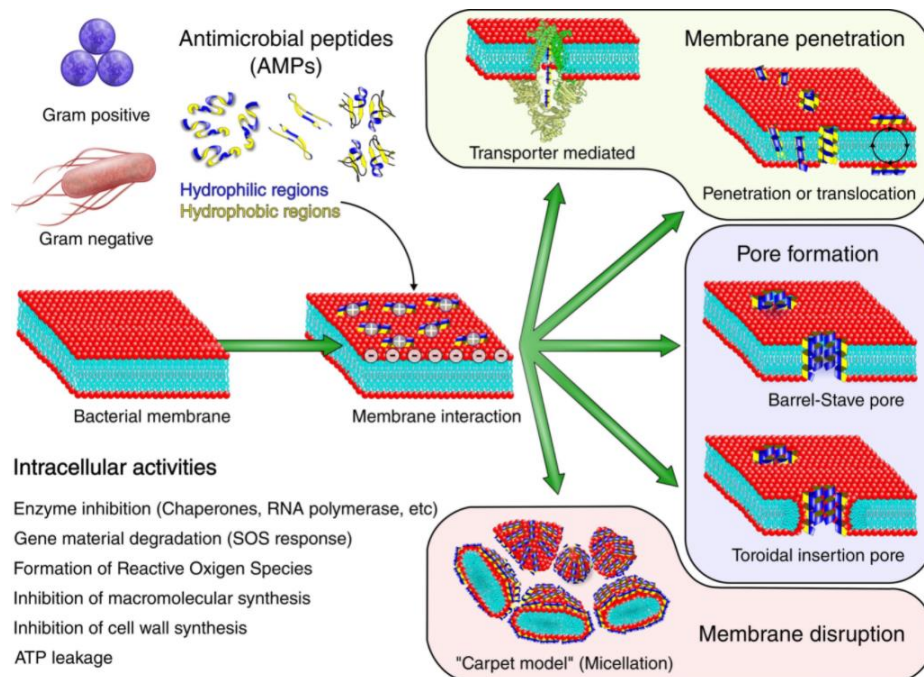
Figure 2.1. The antimicrobial mechanism of AMPs.[20]
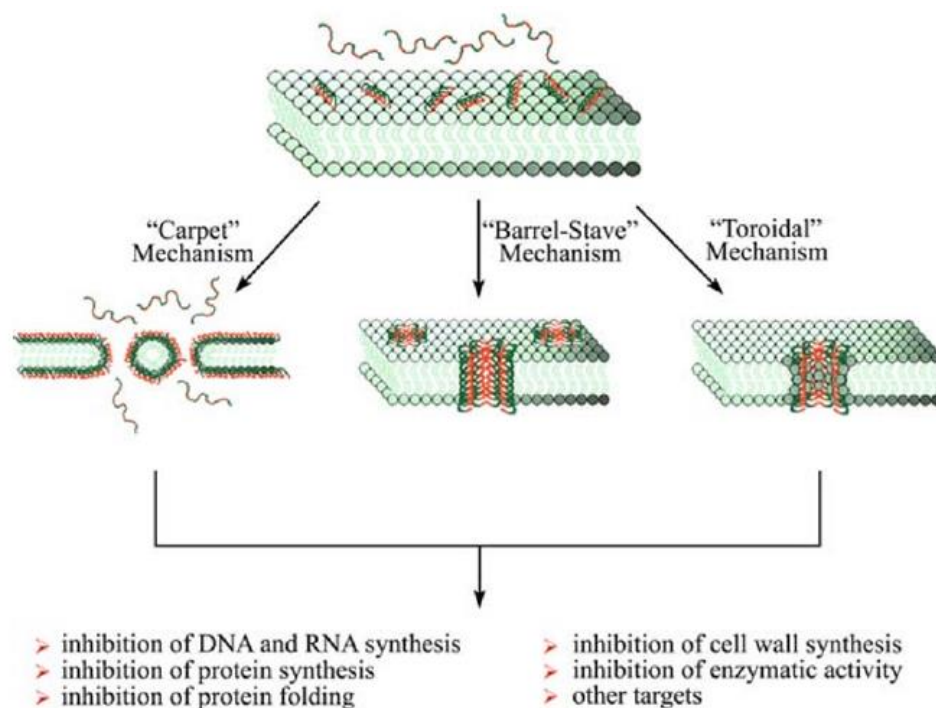
Figure 2.2. Three membrane action models of AMPs.[36]

The first clinical trial of AMPs was conducted in 1939 which was tyrothricin, a mixture of tyrocidine cationic cyclic decapeptides and neutral linear gramicidin.[37] Hitherto, still no significant resistance of tyrothricin was reported after more than 80 years of local

treatment on skin and oral mucosa infection.[38] However, due to its cytotoxicity and hemolysis, the clinical practice of tyrothricin was limited. Whilst distinct from tyrothricin which was the non-resistance inducing antimicrobial, through the incessant evolution and mutation of bacteria, both gram-positive and gram-negative bacterial strains have displayed antimicrobial resistance against other AMPs products.[35, 39]

Moreover, although the study of AMPs can be dated back to nearly 100 years ago, but only several AMPs products managed to complete the required clinical trial phases and became available in clinical prescription, including bacitracin[40], polymyxin B[41], polymyxin E (colistin), tyrothricin[37], gramicidin D[42], gramicidin S[43] and daptomycin[44, 45]. However, not only as peroral antimicrobial medicine in vivo, the AMPs products also could immobilize at the biomaterial surface as the clinical coating of medical instruments (**Figure 2.3**). Hence, the weakness of AMPs could be alleviated, such as the short lifetime (caused by proteolysis) and the low biocompatibility. For instance, in the report of Humblot et al.[46], the antimicrobial activity of the AMPs immobilized monolayer remained active after the stability study of 6 months. Nevertheless, as the lacking of detailed cytotoxicity and hemolysis study for AMPs coating, it still needed to investigate further.[47]
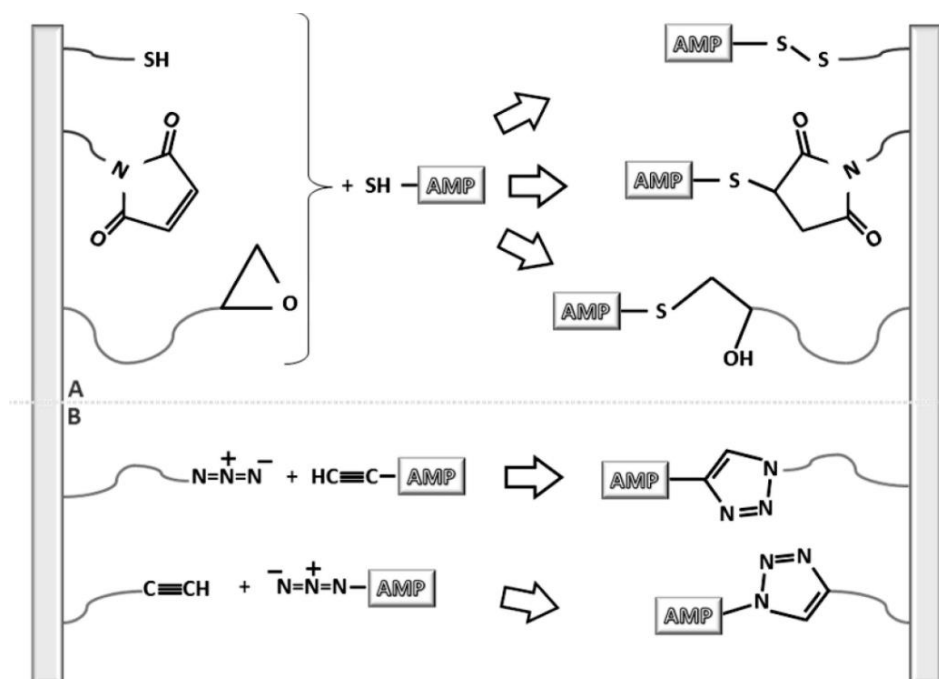
Figure 2.3. The examples of tethering AMPs on material surface and their function prediction.[47]

Due to the cytotoxicity and hemolysis of current AMPs antimicrobial and the extensively existing AMPs databases as mentioned above, some researchers had utilized the computer-aided engineering via high-throughput approaches to discover potential next-generation AMPs antibiotics. The data mining of potential AMPs could be implemented due to its fixed amino acid number and vast possible peptides sequence. For example, **Figure 2.4** performed the process of computational modelling for finding next generation antibiotics.[48] In this study, Cherkasov et al.[49] built two large random short peptides (9 amino acids) libraries and applied an artificial intelligence technique to predict the antimicrobial efficiency thereof. The precision of the prediction was verified by random inspection, as the best peptides construction better antimicrobial efficiency against several MDR strains than conventional antibiotics and showed minimal hemolysis

and cytotoxicity. However, the industrial synthesis of these highly ordered short peptides is limited by the cost yet.
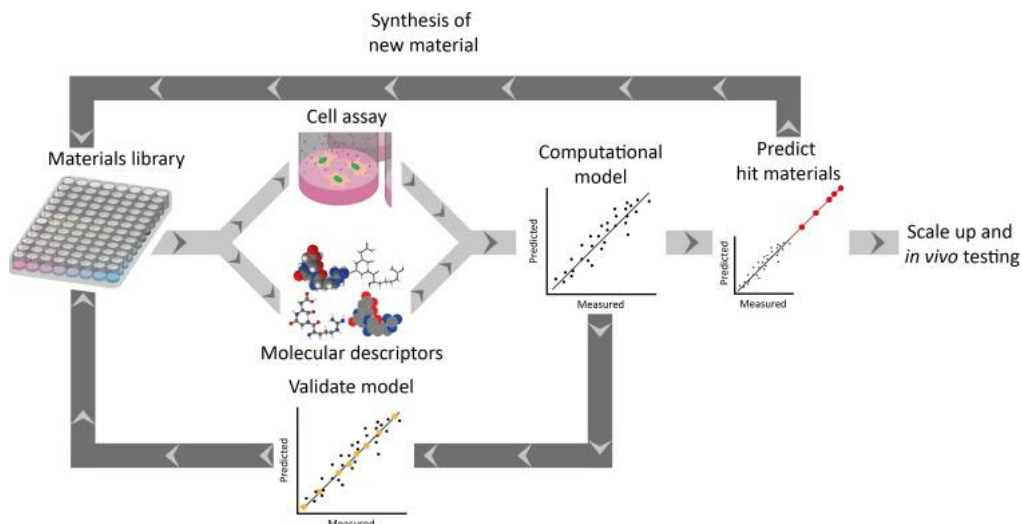
Figure 2.4. The high-throughput screening model for the prediction of novel antimicrobial.[48]

2.2 AMPs Mimicking Polymers

As mentioned above, the limitation of AMPs in pharmacy industry included high production cost, cytotoxicity, and proteolysis. Therefore, it leads to increase research focusing on the synthesis of AMPs mimicking polymers which possess similar structure but better productivity through the utilization of advanced polymerization technique.

The study of synthetic antimicrobial polymers had been carried out for decades. But they were commonly utilized as antimicrobial biomedical material because of their toxicity in vivo.[21, 22] For AMPs mimicking polymers, they had the similar broad-spectrum antimicrobial activity and biocompatibility of AMPs, and more importantly these polymeric mimics had the potential to reduce the hemolysis of AMPs and prevent the development of potential drug-resistance of MDR strains. Also, the proteolysis of AMPs might be overcome through the application of its mimicking polymer due to the difference

10

According to reports[50], there had

been one type of AMPs mimicking polymer (brilacidin or PMX30063, PolyMedix, Inc.,

Radnor, PA, USA, as shown in **Figure 2.5**) which was undergoing phase II clinical trial

against oral mucositis and inflammatory bowel disease on patients.[51, 52]

Brilacidin

Figure 2.5. The chemical structure of brilacidin.[53]

In most studies, the polymer mimics of AMPs showed minimal hemolysis, whereas the

cytotoxicity thereof still needed to be further improved. For example, the copolymer

synthesized by Exley et al. which mimicked the structure of lysine and arginine presented

minimal hemolysis even at high concentration, but low cytotoxicity emerged in MTT

experiments.[54] Consequently, some design guidance of the antimicrobial polymer

construction had been proposed to alleviate the cytotoxicity of the AMPs mimicking

polymer.

In the early study, researchers focused on applying two types of functional groups, the

cationic group, and the hydrophobic group to mimic the amino acid sequence of AMPs.[55, 56] The general accepted theory of these two functional monomers was that the cationic

group could force polymer to adsorb onto the cell membrane by electrostatic adsorption,

where the hydrophobic group could consequently result in the disruption of membrane

wall. However, in the study of Tew et al.[57], by further mimicking the secondary structure

of AMPs (magainin which is facially amphiphilic peptides constructed by β-amino acids),

the reported amphiphilic polymers in which hydrophobic and hydrophilic groups

segregate onto opposite faces of the chain conformation (as shown in **Figure 2.6**) revealed

similar antimicrobial activity with magainin. Even this magainin mimicking polymer

represented unexpected hemolytic activity, this study provided us guidance on the side

chain design by adjusting the charge and hydrophobicity to mimicking AMPs.
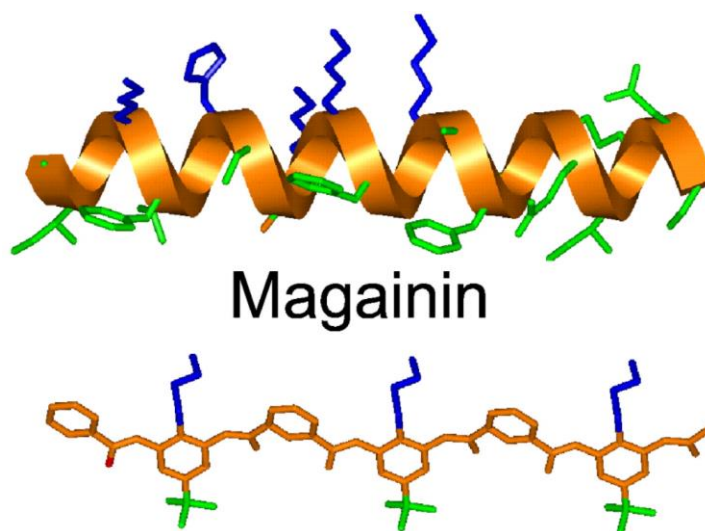
Figure 2.6. The amphiphilic structure of magainin and the polymer mimics thereof.[57]

As described above, the incorporating of hydrophilic monomer units could adjust the

final hydrophobicity of the synthetic polymer to promote the antimicrobial ability and

alleviate the potential cytotoxicity.[58, 59] In light of this, by exploiting the high-throughput

polymerization, a series of PET-RAFT generated polymer libraries with ternary monomer

system (hydrophobic, hydrophilic, and cationic monomers) reported by Judzewitsch et

al.[60], examined that the bacteriostatic efficiency of the final product could be regulated

by the ratio change between the hydrophilic, hydrophobic and cationic monomers which

was showed in **Figure 2.7**. However, the cytotoxicity of this high-throughput synthesis study might be challenging to examine, due to the unpurified residue in each small chamber. Additionally, the DPs and the choice of functional moieties could be tuned to optimize the biocompatibility likewise, whereas some studies had examined the ratio of hydrophilic and hydrophobic groups are more influential factor than targeted DP.[61] To synthesize an AMPs mimicking polymer with efficient antimicrobial activity and biocompatibility, the structure design should notice the balance of the hydrophilic and hydrophobic monomer, given that the typical antimicrobial mechanism of AMPs was the disruption of bacterial membrane based on its hydrophobicity which also resulted in its cytotoxicity. Further, the amphiphilic property of polymers would also influence its behavior in solution (e.g., micellization) which might cause the unwanted hemolysis and hinder the interaction of hydrophobic moieties and bacterial membrane.[62] However, through the segmentation of hydrophobic monomer distribution, the multiblock copolymers could enhance their selectivity toward bacterial membrane, but mammalian cells instead, thereby increasing the biocompatibility thereof.[63]
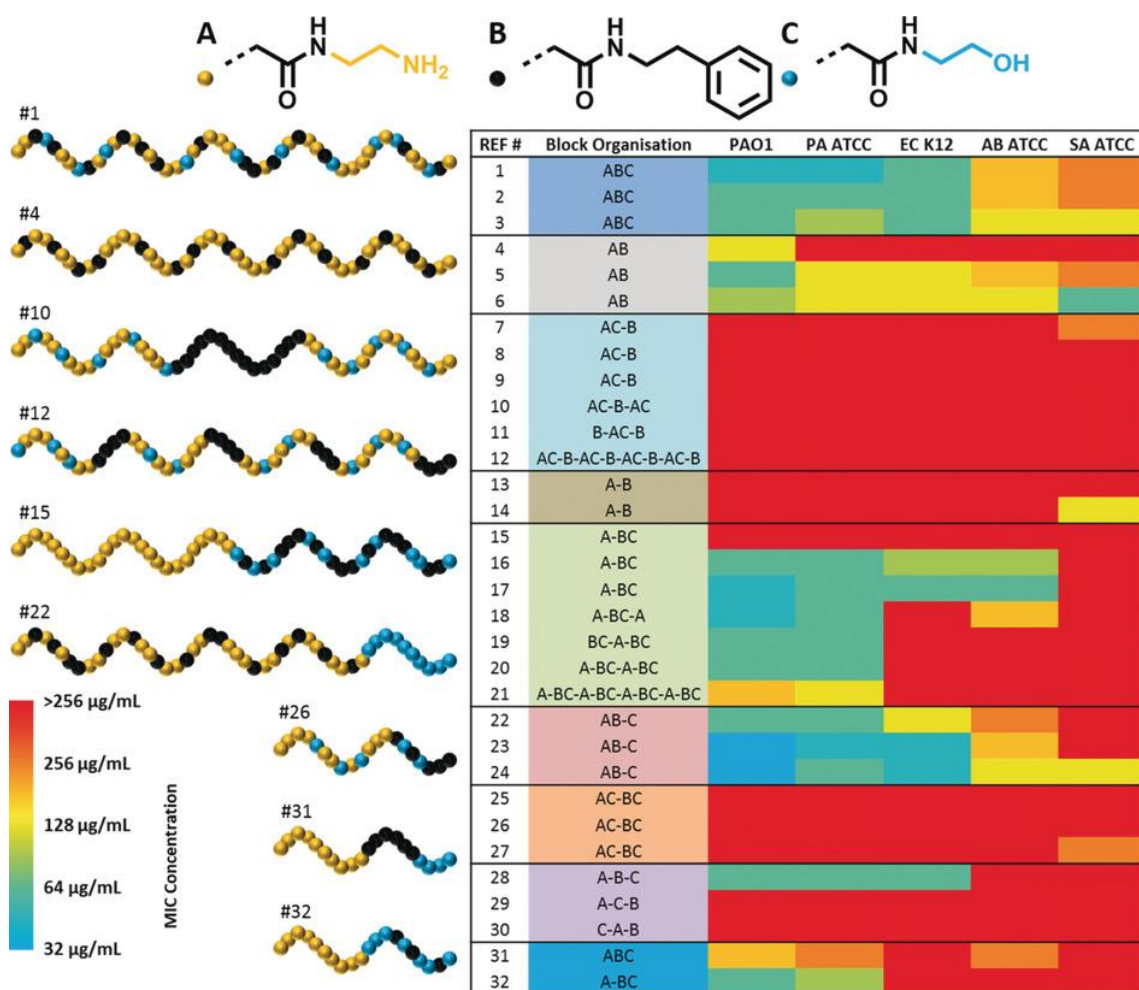
Figure 2.7. The MIC heat map of the polymer library against different bacteria strains.[60]

Moreover, another possible strategy to reduce cytotoxicity was to vary the spatial position of charge and tails. In the study of Lienkamp et al.[51], the amphiphilic pyridinium-methacrylate copolymer could result in higher bactericidal activity but also higher hemolysis when the charge and tail were placed on the separate centers. But when applying the charge and tail on the same center, the hemolysis decreased notably.

Overall, the architecture of AMPs could be mimicked to generate antimicrobial polymers which were economical and less time-consuming than the industrial synthesis of nature AMPs. The defect of synthetic AMPs mimics was also noticeable, as their

14

biocompatibility was relatively lower than conventional antibiotics.[64] However, due to the dose directly determined the toxicity, to reduce the dose of polymer and overcome the antibiotic resistance from bacteria, utilizing AMPs mimicking polymer as adjuvant or synergist could be an appropriate clinical plan to combat MDR strains.

2.3 Synergy

Through the combination therapy, the cytotoxicity from components could be reduced by declining the dose to alleviate the side effects of the coadministration. Thereinto, synergy was the interaction among two or more components which could yield out an equivalent or more efficient pharmacologic action by dropping dosage.[65] According to the model first provided by Chou et al. in 1984[42], the Fractional Inhibitory Concentration Index (FICI) was applied to identify the synergetic degree by using the chequerboard assay as shown in **Equation 2.1.**[66] The synergetic, antagonistic, and adiaphorous FICI correspond to smaller than 0.5, larger than 4.0, and between 0.5 and 4.0, respectively. Next subchapter was the review of several studies about synergy or combination between polymers and antibiotics.

$$FICI = \frac{MIC_1 \ in \ combination}{Individual \ MIC_1} + \frac{MIC_2 \ in \ combination}{Individual \ MIC_2}$$

Equation 2.1. The equation of fraction inhibitory concentration index, $MIC_{1 \ or \ 2}$ represents the MIC of component 1 or 2 in combination therapy

2.3.1 Synergy of Antimicrobial Peptides

As the crucial advantage of AMPs is their low resistance inducing antimicrobial property through the multi-targeting mechanism instead of one specific receptor,[67] the combination therapy of AMPs and commercial antibiotics could further eliminate the MDR bacterial infection, reduce the clinical dosage and alleviate the side effects. Recently, several reports have reviewed the idiographic synergy between AMPs and other traditional antibiotics and demonstrated the limitation and advantages of these combination therapies in clinical.[68-70] Specifically, these AMPs which show synergistic interaction with antibiotics include the traditional AMPs that have widely engaged in production (such as colistin[71], daptomycin[72], nisin[73], polymyxin B[74], polymer E[75], and protamine[76]) and the newfound AMPs (such as FK-13[77], LL-37[78], melimine[79], magainin II[80], and SET-M33[81]).

2.3.2 Synergy of Antimicrobial Polymers against Gram-negative Strains

As the key target of AMPs, the membrane structure of gram-negative strains was divided into 3 layers, including the outer membrane, the cell membrane, and the peptidoglycan layer between them. Although the peptidoglycan layer of gram-negative was much thinner than gram-positive pathogens, the outer membrane of gram-negative bacteria could limit the invasion of hydrophobic substances which was caused by the tight arrangement of the glycerophospholipids on the outer membrane.[82] Therefore, several combination therapy studies between polymers and antibiotics have been conducted against gram-negative pathogens.

Firstly, the antibiotics of tetracycline class were prevalent choices in combination therapy for the synergy with polymer. In the study of Ng et al.[23], the vitamin E contained

cationic polycarbonates had high synergy (FICI with doxycycline = 0.11) with doxycycline, streptomycin, and penicillin G against both gram-negative and positive bacteria and had satisfactory biocompatibility results in the hemolysis test of red blood cells. Thereinto, doxycycline showed the lowest FICI value against *P. aeruginosa* which was attributed to the enhanced cell membrane permeability of antibiotics through vitamin E re-balanced amphiphilicity of polymers. Additionally, images taken from SEM and confocal microscope showed that the slight membrane disruption would arise from the increasing concentration of this polymer, as small pores formed, and permeability increased. Interestingly, in this study, the susceptibility of *P. aeruginosa* to penicillin G was recovered under the assistance of this polymer since penicillin G presented low bactericidal effect on *P. aeruginosa*. Furthermore, this study could further investigate if the same bactericidal efficacy was achieved when targeting MDR strains, as to identify the increased membrane permeability and heightened the influx of conventional antibiotics whether could overcome the drug-resistant species. **Figure 2.8** presented the chemical structure of this vitamin E containing cationic polycarbonates and the confocal microscope images of *P. aeruginosa* under the influence of antibiotics alone or coadministration.
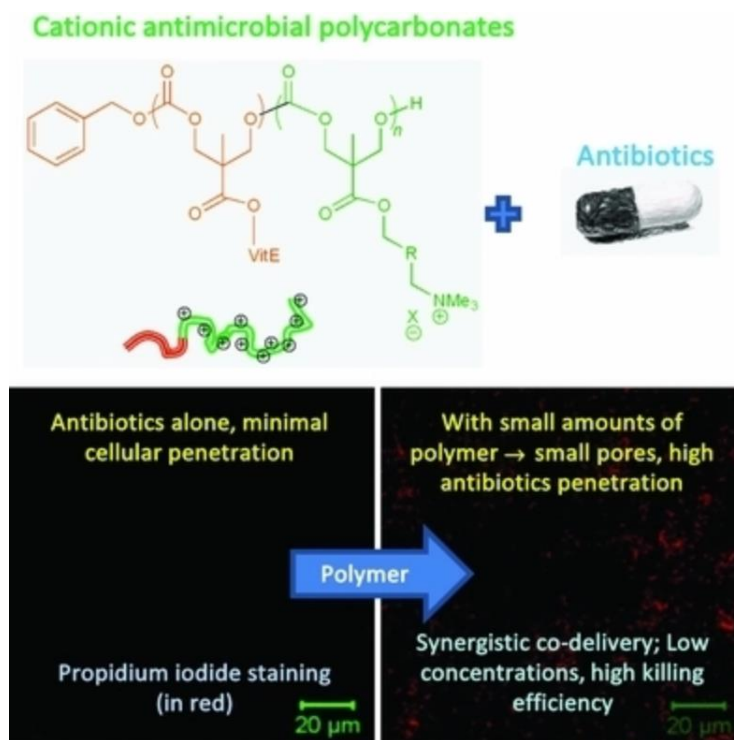
Figure 2.8. The vitamin E containing cationic polycarbonate and its brief synergy mechanism.[23]

In another study, Namivandi-Zangenh et al. polymerized a peptide mimicking polymer by RAFT and evaluated its synergetic effect with a range of antibiotics against *P. aeruginosa*.[28] From the results, the colistin and doxycycline presented synergy with this ternary copolymer according to the block figure by checkboard assay in **Figure 2.9**. Additionally, the combination of doxycycline and synthetic polymer could overcome the resistance when testing against MDR *P. aeruginosa* PA32 and PA37, the isolates from patients with microbial keratitis. In the biofilm killing kinetic study, the bactericidal degree against *P. aeruginosa* PAO1 reached more than 99% by applying doxycycline as synergists. Inspired by this, doxycycline could be explored further in future synergistic study, as the synergy between polymer and antibiotics was only evaluated through a binary combination in this work. Thus, the combination of doxycycline, colistin, and

polymer could be another possible ternary combination that reduced the dosage of the antibiotics further as presented in Chapter 3 herein.
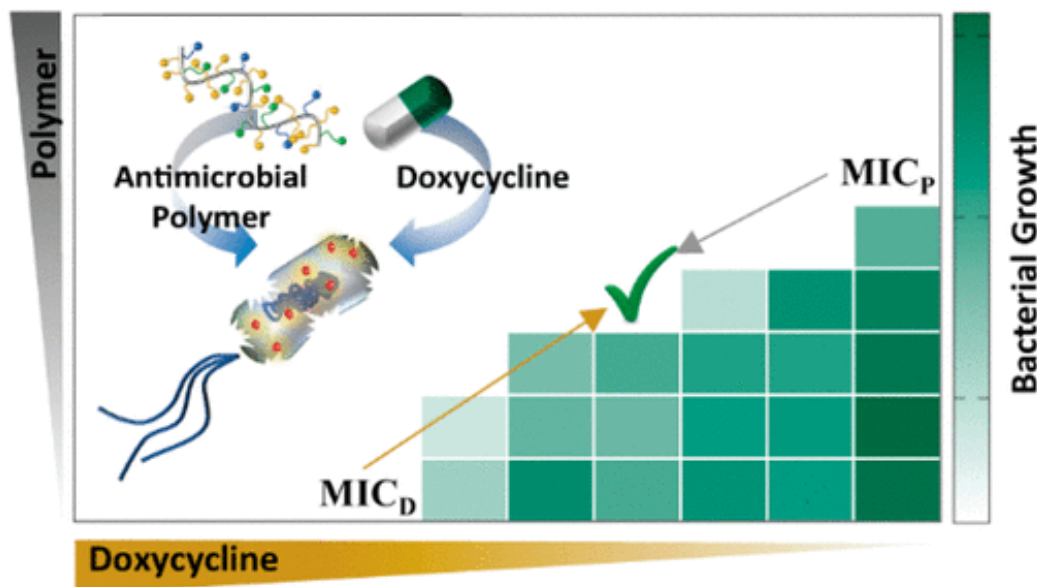
Figure 2.9. The synergetic assay between ternary polymer and doxycycline.[28]

Dendrimers were another class of antimicrobial polymers which had been widely reported as drug delivery systems. Nevertheless, this different molecular structure might have a different co-action mechanism with conventional antibiotics compared with the chain-typed antimicrobial copolymers. For instance, the maltose modified dendrimers of poly(propylene imine) showed plausible antimicrobial effect when combined with nadifloxacin against *E. coli*, *P. aeruginosa* and *P. hauseri* in the study of Felczak.[83] During the cytotoxicity evaluation study on several different eukaryotic cells lines, the results showed the modification of maltose on this polymer could facilitate biocompatibility. As shown in **Figure 2.10**, the combination therapy of this maltose-modified dendrimers and nadifloxacin could moderate the cytotoxicity of this dendrimer.
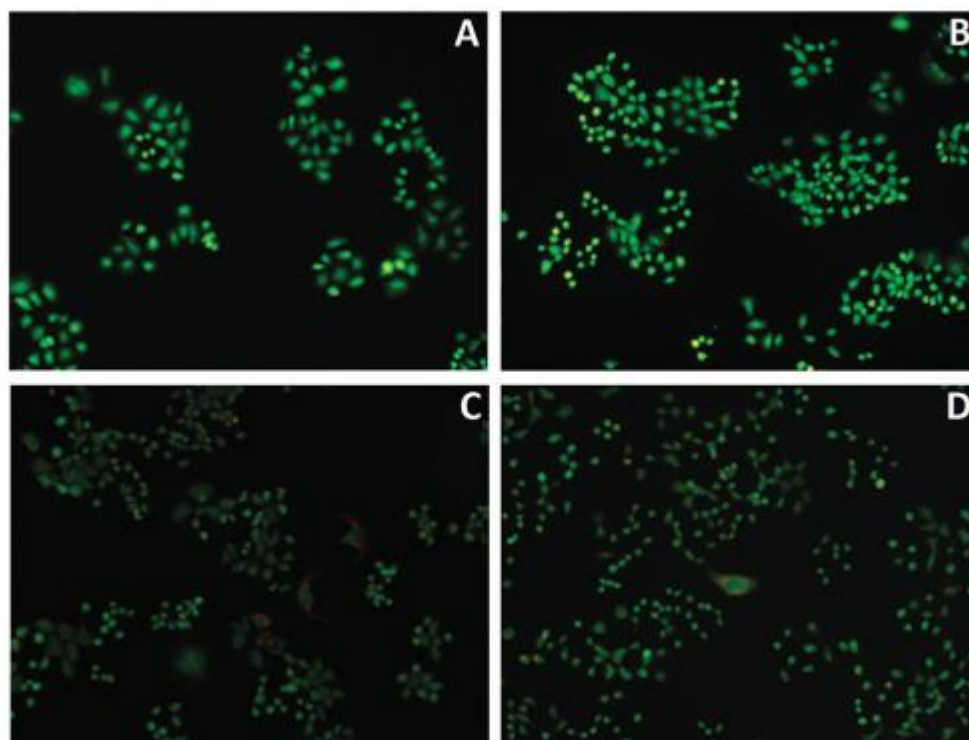
Figure 2.10. The fluorescent microscopy image of Neuro 2a cell line with acridine orange and ethidium bromide, (A) control group, (B) nadifloxacin 5 mg/mL, (C) maltose-modified dendrimer 3 mM, (D) nadifloxacin 5 mg/mL and maltose-modified dendrimer 3 mM.[83]

Another feasible synergetic combination of AMPs mimicking polymer was AMP which both were membrane disrupting antimicrobials. The probability of synergy between them might be high because of the recent study by Typas groups[84] which proposed that the synergy was more prevalent for antimicrobials that shared the same type of mechanism. For example, the cationic conjugated polymers reported by Tian et al. performed synergetic activity with antimicrobial peptides against *E. coli*.[85] Further, against kanamycin-resistant *E. coli*, this polymer also had synergy with polymyxin B and polymyxin E which are supposed to be the membrane disrupter. Interestingly, in the CLSM and SEM study, the membrane disruption was only observed when inducted with the combination of polymer and polymyxin, but no obvious morphological change with

the individual component at the same concentrations in combination. This phenomenon indirectly revealed the coadministration mechanism theory that the influx of antibiotics would be increased through enhancing cell membrane permeability under the assist of AMPs. The brief mechanism of this study was presented in **Figure 2.11**. Inspired by this combination, the AMPs (e.g., colistin, polymyxin B, nisin) and the polymer mimics thereof could be investigated further as the binary combination of the two types membrane disruptors might facilitate the global antimicrobial activity.
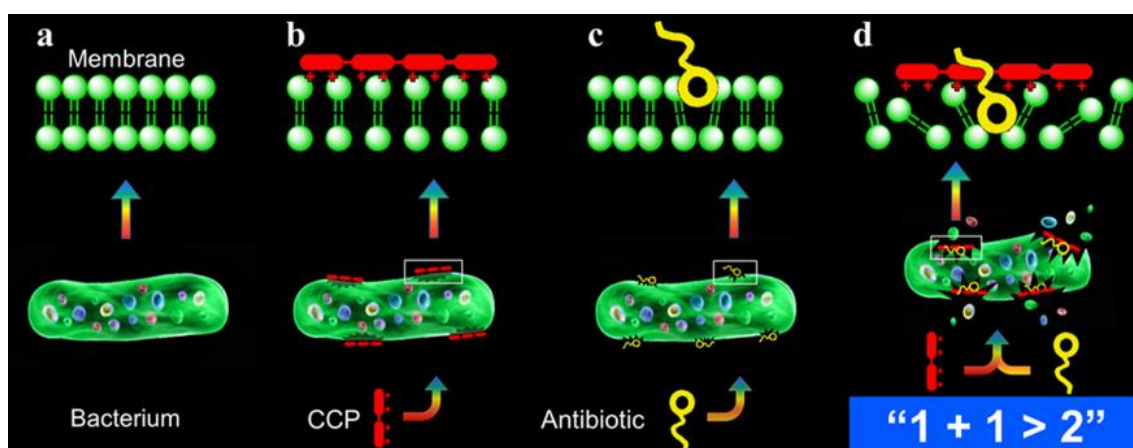
Figure 2.11. The synergetic mechanism between polymers and antibiotics, (a) bacteria, (b) bacteria + polymer, (c) bacteria + antibiotic, (d) bacteria + polymer + antibiotic.[85]

Furthermore, the antibiotics of the rifamycin family which act as the RNA synthesis inhibitors are another interesting object to investigate the synergy with AMPs or the polymeric mimics thereof. In the study of Ding et al. in 2020, the guanidium-functionalized polycarbonate (pEt_20) could reverse the resistance of MDR *A. baumannii*, through the synergy of rifampicin or auranofin.[24] Specifically, the minimum bactericidal concentration of rifampicin could be decreased 4096-fold with pEt_20 which was 256-fold stronger than the combination of rifampicin and colistin against MDR *A. baumannii*.

In hemolysis, cytotoxicity, and MDR *A. baumannii* mouse model test, the synergetic combination of pEt_20 and rifampicin showed better biocompatibility than individual usage of colistin or rifampicin. This synergetic combination possessed potential to alleviate the nephrotoxicity of colistin. **Figure 2.12** demonstrated the detailed membrane penetration mechanism and the recession of the MIC and MBC value.
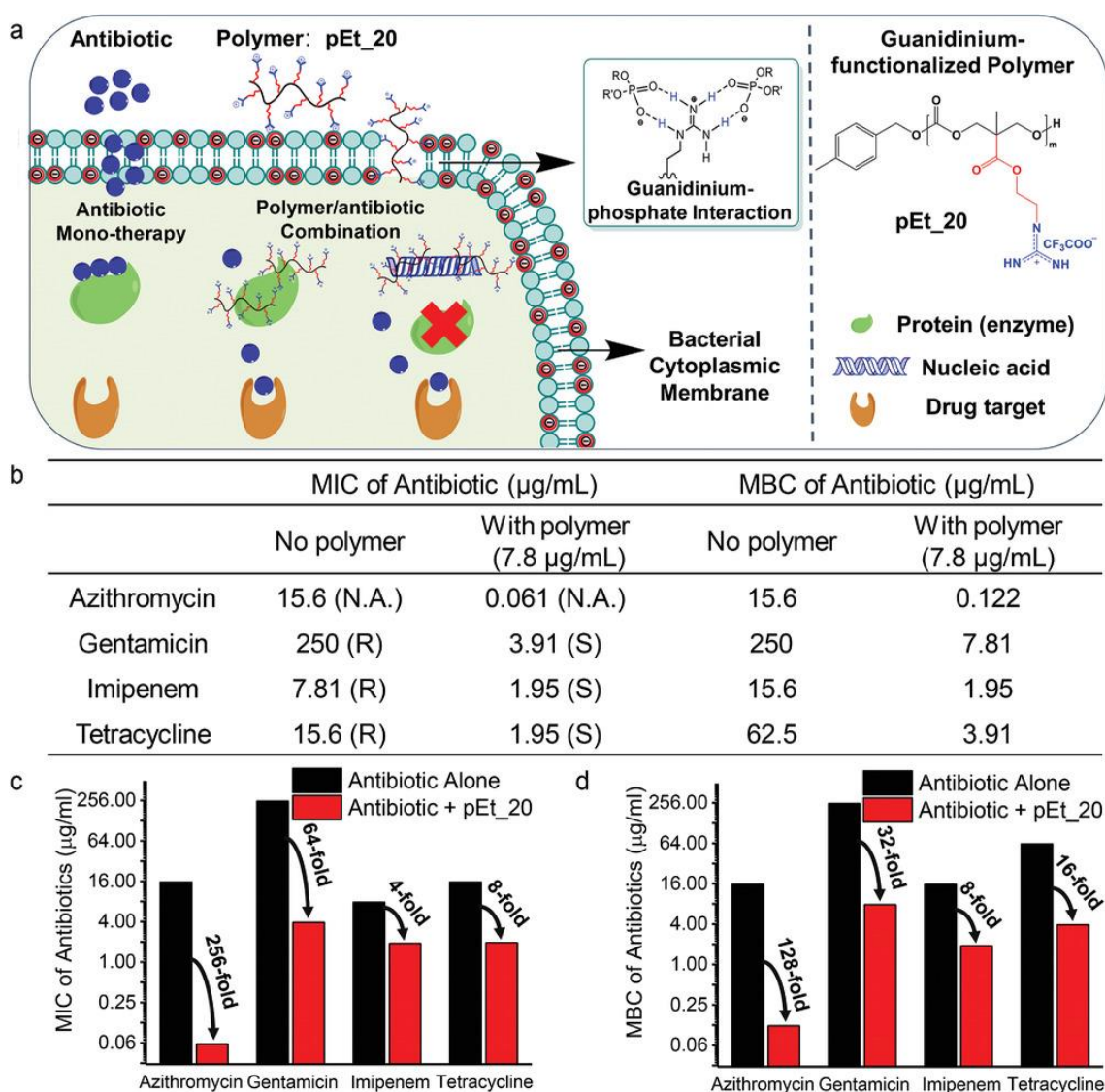
Figure 2.12. The combination therapy of traditional antibiotics and polymer pEt_20 against *A. baumannii*, (a) membrane penetration mechanism of polymer pEt_20, (b) MIC and MBC of antibiotics with or without pEt_20, (c) MIC recession when combined antibiotics and pEt_20, (d) MBC recession when combined antibiotics and pEt_20.[24]

Generally, the membrane permeability change was an important characteristic in the synergetic mechanism between antimicrobial polymers and commercial antibiotics. Therefore, to target the enhancement of membrane permeability for gram-negative bacteria, Khalil et al.[86] emplyed polyethyleneimine to co-administrate with 16 different antibiotics against *P. aeruginosa*. This polymer presented low FICI value combined with cephalosporins, rifamycin, and novobiocin. The sub-inhibitory concentration (250 μg mL^{-1}) of the polymer could decrease the MIC value of antibiotics by more than 40-folds. The detailed MIC, FICI, and interaction evaluation were presented in **Table 2.1**. On the other hand, this polymer also inhibited the bacterial killing efficiency of polymyxin and aminoglycoside which might arise from competing binding of the bacterial cell membrane by either antibiotics or polymers.

Table. 2.1. The individual MIC and FICI of the co-administration between antibiotics and polyethyleneimine against *P. aeruginosa*. ND, not determined.[86]

Antibiotic class	Antibiotics	MIC (μg mL^{-1}) with		FICI index	Interaction	Killing efficiency
		Antibiotic alone	Antibiotic and polymer (250 μg mL^{-1})			
Novobiocin	Novobiocin	1,600	140	0.01	Synergy	Synergy
Aminoglycosides	Tobramycin	50	240	4.81	Antagonism	Indifference
	Kanamycin A	1,600	1,920	1.21	Indifference	ND
	Gentamicin	1,120	1,920	1.72	Indifference	ND
Tetracyclines	Tetracycline	20	20	1.00	Indifference	ND
Cephalosporins	Ceftazidime	400	10	0.04	Synergy	Synergy

	Cefotaxime	960	120	0.14	Synergy	ND
Chloramphenicol	Chloramphenicol	100	40	0.41	Synergy	Synergy
Macrolides	Erythromycin	140	140	1.00	Indifference	ND
Polymyxins	Polymyxin E	5	10	2.01	Antagonism	ND
	Polymyxin B	5	10	2.01	Antagonism	Antagonism
Rifampicin	Rifampin	20	10	0.05	Synergy	Synergy
Cationic glycopeptides	Vancomycin	1,120	3,800	3.40	Antagonism	Antagonism
Fluoroquinolones	Ciprofloxacin	30	30	1.00	Indifference	Indifference
	Ofloxacin	10	10	1.00	Indifference	ND
	Norfloxacin	15	10	0.68	Indifference	ND
ß–Lactams	Ampicillin	2,240	100	ND	ND	Synergy
	Carbenicillin	2,240	60	ND	ND	Synergy
	Piperacillin	40	7.5	ND	ND	Synergy
	Ticarcillin	1,120	20	ND	ND	Synergy

Except for combination therapy, antibiotics could be incorporated into the monomer system and subsequently polymerized. In the study of He et al.[87], they innovatively added ciprofloxacin into the cationic and hydrophobic monomer system as shown in **Figure 2.13**. And this copolymer showed a better MIC value than the polymer without ciprofloxacin monomers against *E. coli*. Considering the only bacterial strain in this study is *E. coli*, to increase the versatility of this copolymer, diverse types of bacteria must be tested and the biocompatibility study thereof also had not been identified.
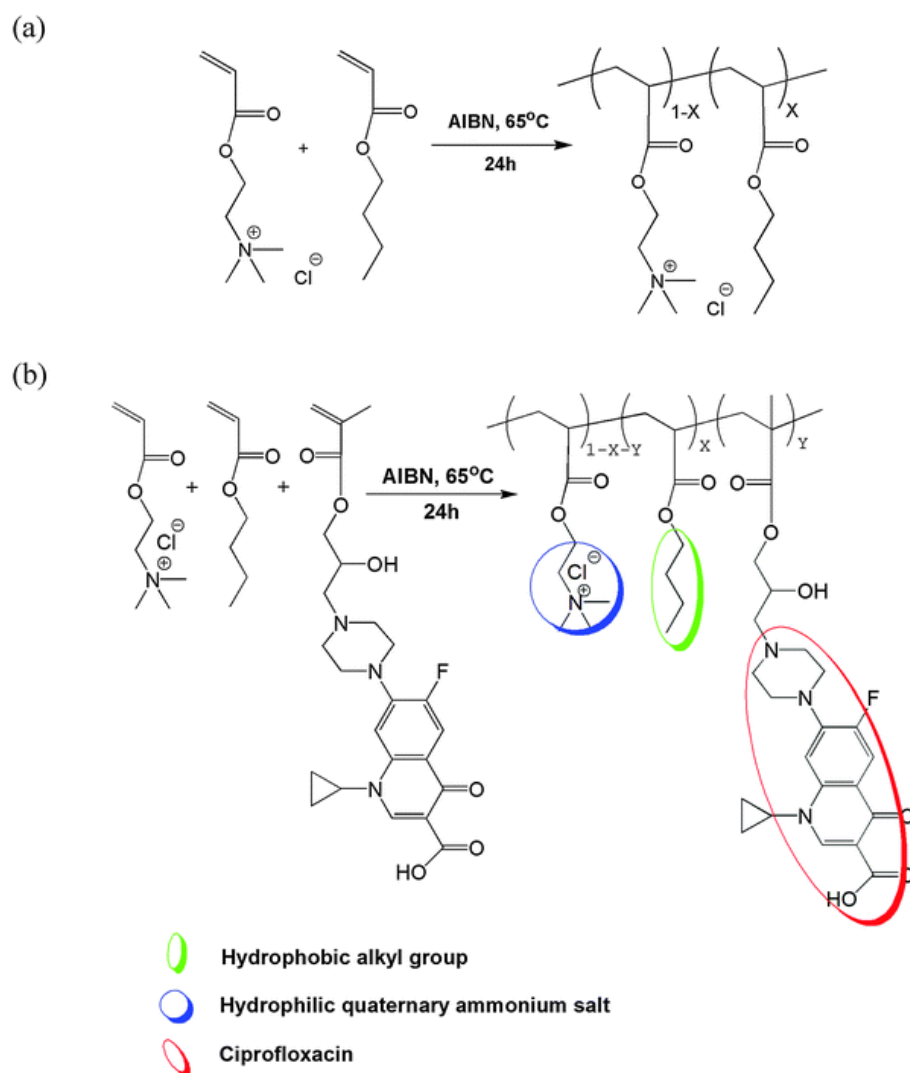
Figure 2.13. The synthesis of antimicrobial ciprofloxacin modified polymer.[87]

2.3.3 Synergy of Antimicrobial Polymers against Gram-positive Strain

The MDR bacterial infection was an urgent worldwide healthcare problem, which is a continuous threat to human life. Thereinto, the gram-positive strain Methicillin-Resistant *S. Aureus* (MRSA) occupied nearly one-third of the MDR infectious patients.[88] For instance, the MRSA strain USA$_{300}$ which was first reported in 1999 at a jail in Mississippi (USA) was widely spread in a decade that had become the dominant pathogen of superficial skin and soft-tissue infections and even implicated the lethal septicaemia and

25

endocarditis.[89-93] Until 2011, the USA_{300} had been the most common hospital and community acquired MRSA isolates which increased the clinical burden and kept threatening human health.[94] Furthermore, in contrast with gram-negative strain, the peptidoglycan of gram-positive strain which occupied 40% of the cell mass, was thicker than gram-negative bacteria and directly exposed on the cell membrane surface.[95] Therefore, when against the epidemic of gram-positive pathogens, such as MRSA, the design of antimicrobial polymer construction should be targeted due to different membrane structures. Additionally, the coadministration of these antimicrobial polymers and antibiotics should be more specific and narrow-spectrum combination due to the weaker susceptibility of broad-spectrum antibiotics against MDR gram-positive strains. For example, in the study by Thappeta et al.[96], the cationic AMPs mimics which synthesized by chitosan and lysine had synergetic effect with oxacillin and vancomycin against *E. faecalis*. However, the same combination only presented adjuvant effect (0.5 < FICI < 1) against another type of gram-positive strain, *S. aureus*. Moreover, this polymer also presented synergy against gram-negative strain (*E. coli*), but with different antibiotics (streptomycin and tetracycline). The CFU reduction resulted from these combinations were presented in **Figure 2.14**. Hence, the AMPs mimicking antimicrobial polymer might possess wide-spectrum synergy with different antibiotics against different pathogens, but each individual combination might be narrow-spectrum antimicrobials that only aimed at the specific bacteria. Furthermore, in the drug-resistance study, the MIC of this polymer would not increase more than 2 times after co-culture with sub-inhibitory concentration

which revealed the none specific resistance arise. Interestingly, the slight polymer resistance of MRSA had a converse impact which led to the increasing susceptibility of ß-lactam antibiotics, such as oxacillin and carbenicillin. This transition restored the sensitivity of oxacillin and carbenicillin against MRSA and might be due to the antibiotic resistance of bacterial being hindered by the polymers. Even though, the hemolysis and cytotoxicity of the combination had not been examined, these were crucial clinical characteristics where the sole polymer had minimal toxicity toward mammalian cells.
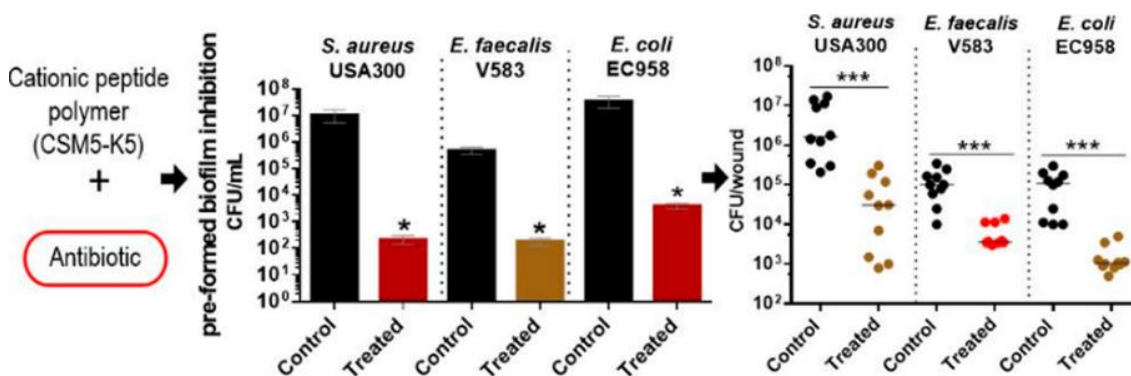
Figure 2.14. The CFU reduction of the synergy between cationic polymer and antibiotics.[96]

Another type of cationic branched polyethyleneimine with negligible toxicity was reported to have synergy with β-lactam against MRSA whose average cell size was significantly increased to inhibit the septa forming under the combination therapy. The bactericidal activity could be achieved when co-administrated with oxacillin. The antibacterial mechanism behind (in **Figure 2.15**) was attributed to the electrostatic interaction between cationic polymer and wall teichoic acid on the cell wall of gram-positive bacteria. The wall teichoic acid is crucial to the localization of cell wall protein. Through the contrast with the wall teichoic acid knockout model, this hypothesis of mechanism was examined further in this study.[97]
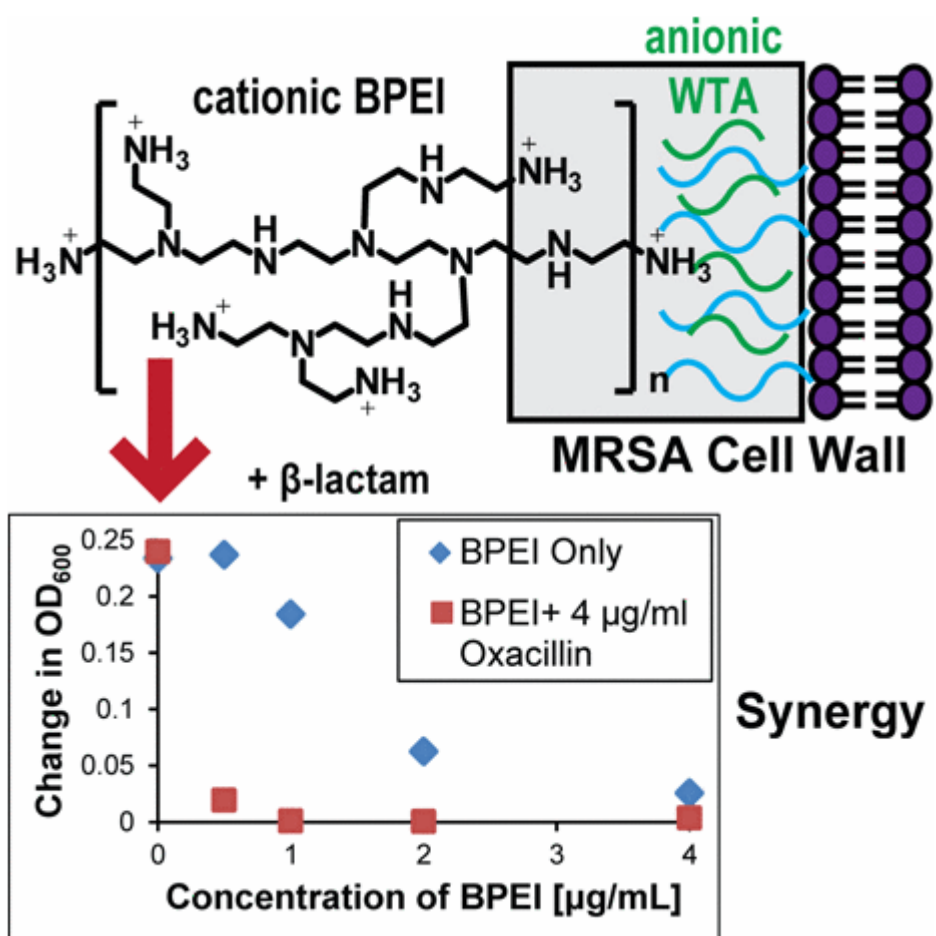
Figure 2.15. The brief mechanism and bacteriostatic activity of the branched polyethyleneimine. The change in OD_{600} (optical density at 600 nm) reflected the cell growth of MRSA after 20 h.[97]

Nonetheless, the research around the synergy between antimicrobial polymer and conventional antibiotics which directly relating AMPs mimicking polymer was still lacking. Next, the synergy of other types of antimicrobial polymer was reported.

Metallopolymer was another focused potential next-generation antimicrobial. In the study of Zhang et al.[98], metallopolymers could act as a reservoir of ß-lactam antibiotics which also had synergetic effects with the released antibiotics against MRSA, as shown in **Figure 2.16**. The membrane disruption could be observed in **Figure 2.17** by SEM. The
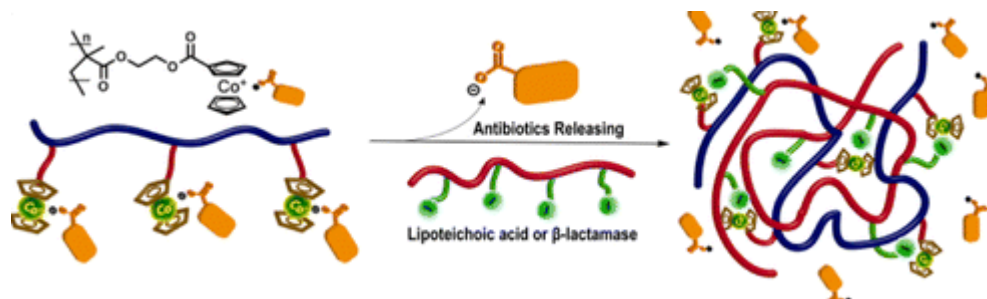
Figure 2.16. The releasing of β-lactam from metallopolymer.[98]
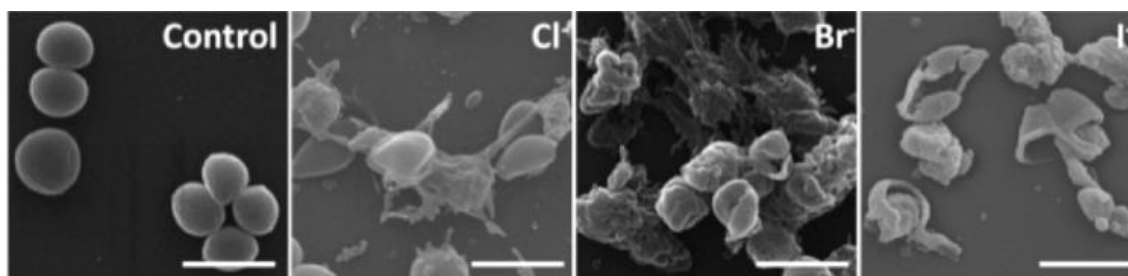
Figure 2.17. The SEM images of Cl⁻, Br⁻, and I⁻ paired metallopolymer and the control group after incubation with MRSA.[98]

In addition to metallopolymers, the drug-load metal nanoparticle could be another choice of combination as well. Meeker et al.[99] synthesized a polydopamine-modified nanoparticle loading with daptomycin which targeted at *S. aureus* through the specific bonding with bacteria surface protein (**Figure 2.18**). It was demonstrated the co-action of the lethal photothermal effect from the polymer and controlled releasing of loaded antibiotics would have a synergetic bacterial killing effect. The loaded natural lipopeptide antibiotic, daptomycin (**Figure 2.20**) could insert into the cell membrane and cause rapid depolarization to inhibit bacterial metabolism.[100] It was worthwhile to note that the synergy might also exist in the combination of daptomycin and its polymeric mimics

Figure 2.19. The chemical structure of daptomycin.[100]

Interestingly, besides the AMPs, other natural compounds have garnered more attention

to identify their possible synergistic capability. According to the traditional Chinese

couplet medicines, Li et al.[101] combined derivates of the radix scutellariae and coptidis

rhizome to generate two types of self-assembled nanostructures (**Figure 2.20**) which

showed a better antibacterial ability than the single nanoparticles to combat *S. aureus*.

Furthermore, the derivates from curcumin (**Figure 2.21**) also showed synergy when

combined with ciprofloxacin against gram-positive bacteria[102]. Inspired by these studies, the traditional combinational recipe of herbal medicine could provide some hints for the potential synergy against MDR bacteria.
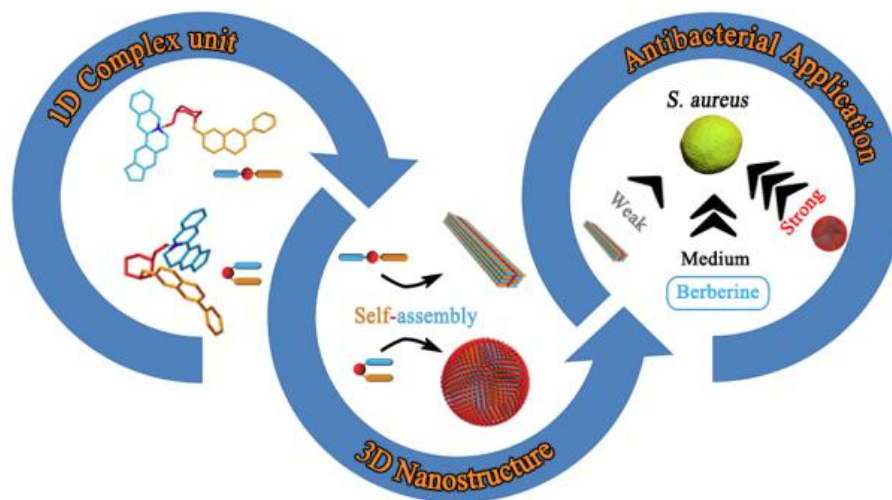
Figure 2.20. The self-assemble and antimicrobial mechanism of the berberine based nanostructure.[101]
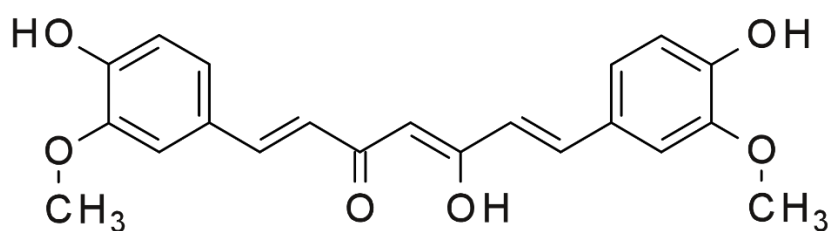
Figure 2.21. The chemical structure of curcumin.

2.3.4 Synergy of Antimicrobial Polymers against Mycobacterium

The mycobacterium, *M. tuberculosis* is another crucial global healthcare issue that results in more than 1.5 million death (2018) annually which has also shown the co-infection possibility with COVID-19 under the worldwide pandemic and accounted for one of the most lethal infection, even more severe than HIV or AIDs.[103, 104] The typical treatment of tuberculosis infection needed combination therapy with more than four traditional antibiotics and lasted half a year due to the broad-spectrum drug-resistance.[105] From the

first antituberculosis combination therapy, the streptomycin and para-amino-salicylic acid in 1950, the investigation of the synergetic prescription or the novel antituberculosis agents had never stopped.[106-109] Since mycobacterium species had developed resistance to nearly all types of commercial antibiotics, the AMPs became important next-generation anti-mycobacterium agents to combat the MDR mycobacterium strains. The potential synergy of AMPs with conventional antibiotics could reduce the dosage of typical antituberculosis therapy further. For example, the glutathione (GSH) was a tripeptide which was well known for its antioxidation function. It also could be applied against mycobacterium. In a recent report, the precursor of GSH, *N*-acetyl cysteine revealed synergy with rifampicin and isoniazid against *M. tuberculosis*, the CFU results of the combination were showed in **Figure 2.22**.[110]
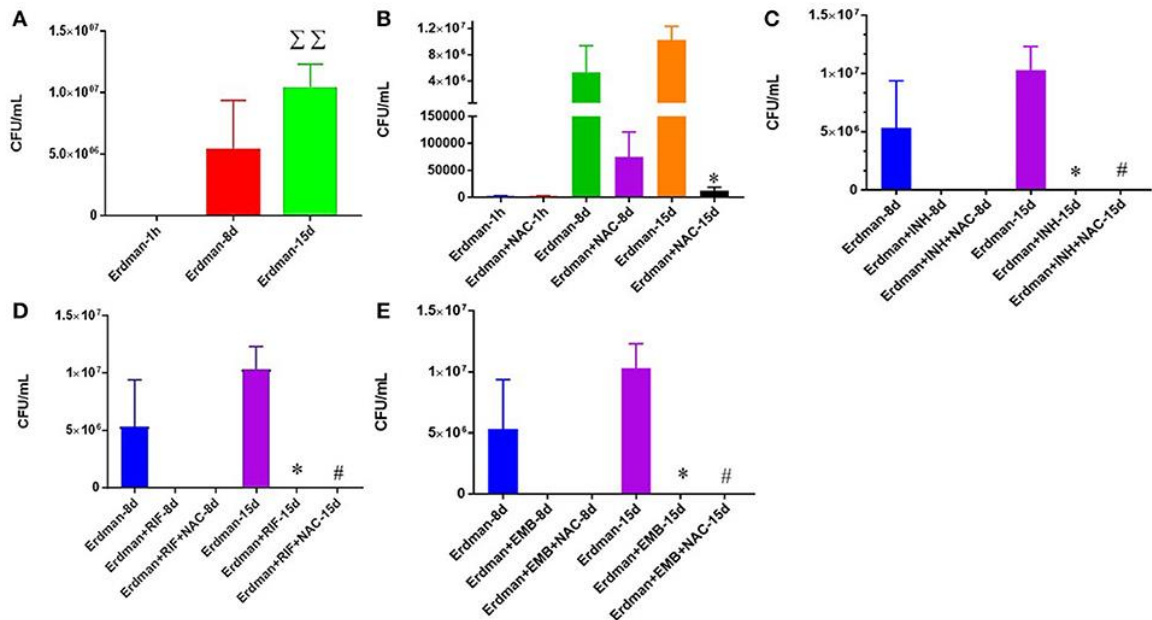
Figure 2.22. The bacterial concentration (CFU) at different time points under the combination therapy between *N*-acetyl cysteine (NAC), isoniazid (INH), ethambutol (EMB), and rifampicin (RIF) against Erdman (*M. tuberculosis* strain), (A) control group, (B) NAC, (C) INH and INH + NAC, (D) RIF and RIF + NAC, (E) EMB and EMB + NAC. *$p < 0.05$ when comparing samples to their respective controls. #$p <$

Additionally, the peptides reported by Khara et al.[111] which were α-helical and hydrophobic amino acids could combat *M. smegmatis* and have synergy with rifampicin. It also demonstrated the hydrophobicity and the α-helicity of the AMPs mimics directly influenced the anti-mycobacterial ability through inducing the rapid membrane depolarization, membrane disruption, and cytoplasm leakage which guided the synthesize of antimicrobial polymers. These above-mentioned antimycobacterial mechanisms of *M. smegmatis* were investigated through several different assays regarding membrane disruption. SEM images of the peptide treated and untreated *M. smegmatis* are presented in **Figure 2.23**.
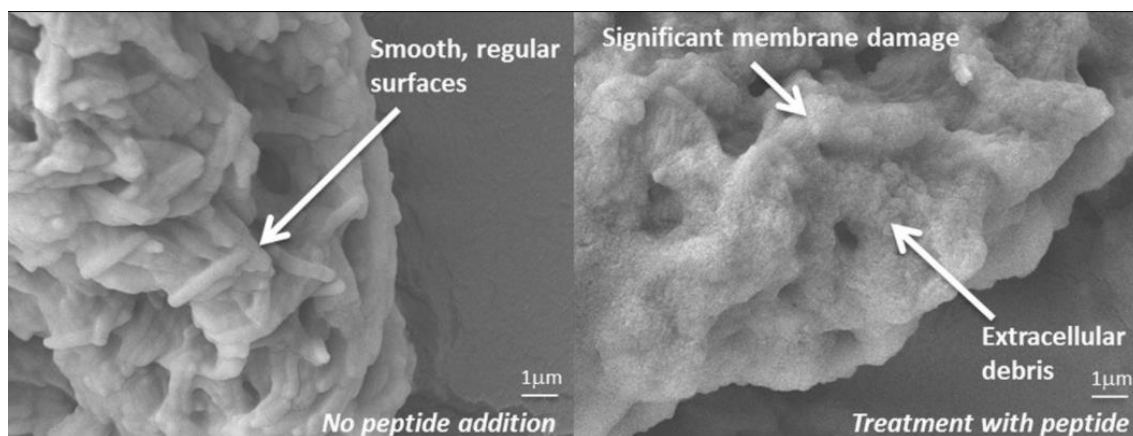
Figure 2.23. The untreated (left) and the treated (right) *M. smegmatis* SEM image.[111]

Furthermore, in the study of antimycobacterial AMPs by Gupta et al.[112], they evaluated and analyzed several natural antimicrobial peptides against *M. smegmatis*. The natural AMPs existed in synergy with rifampicin and polymyxin B. The synergy with the first-line oral drugs, rifampicin directly reduced the clinical dose. But it still needed further

work relating to the drug-resistant species, such as *M. tuberculosis*. The detailed MIC and FICI was showed in the following **Table 2.2**.

Table 2.2. The individual MIC and FICI of the synergy between antibiotics and AMPs.[112]

Antibiotics	AMPs	MIC (μg mL^{-1})				FICI
		Individual		In Combination		
		Antibiotics	AMPs	Antibiotics	AMPs	
Rifampicin	ATRA–1	3.9	31.3	1.95	3.9	0.56
Rifampicin	hBD3–Pepe 4	3.9	62.6	1.95	3.9	0.56
Rifampicin	LL–37	3.9	31.3	0.97	3.9	0.32
Rifampicin	mCRAMP	3.9	15.6	0.97	1.95	0.35
Polymyxin B	ATRA–1	7.8	31.3	1.95	3.9	0.37
Polymyxin B	hBD3–Pepe 4	7.8	62.6	1.95	7.8	0.37
Polymyxin B	LL–37	7.8	31.3	1.95	3.9	0.37
Polymyxin B	mCRAMP	7.8	15.6	1.95	3.9	0.5

There had been several studies demonstrating that the AMPs mimicking polymer could combat mycobacterium. Through high through-put synthesis of libraries of antimycobacterial polymer by varying monomer construction, Judzewitsch et al.[113] provided an efficient manner to investigate novel antibiotics against MDR mycobacterium species. The cationic groups in their ternary monomer system displayed an important role against *M. smegmatis*. The monomer construction of the antimicrobial polymer libraries by high through-put polymerization was showed in **Figure 2.24**.
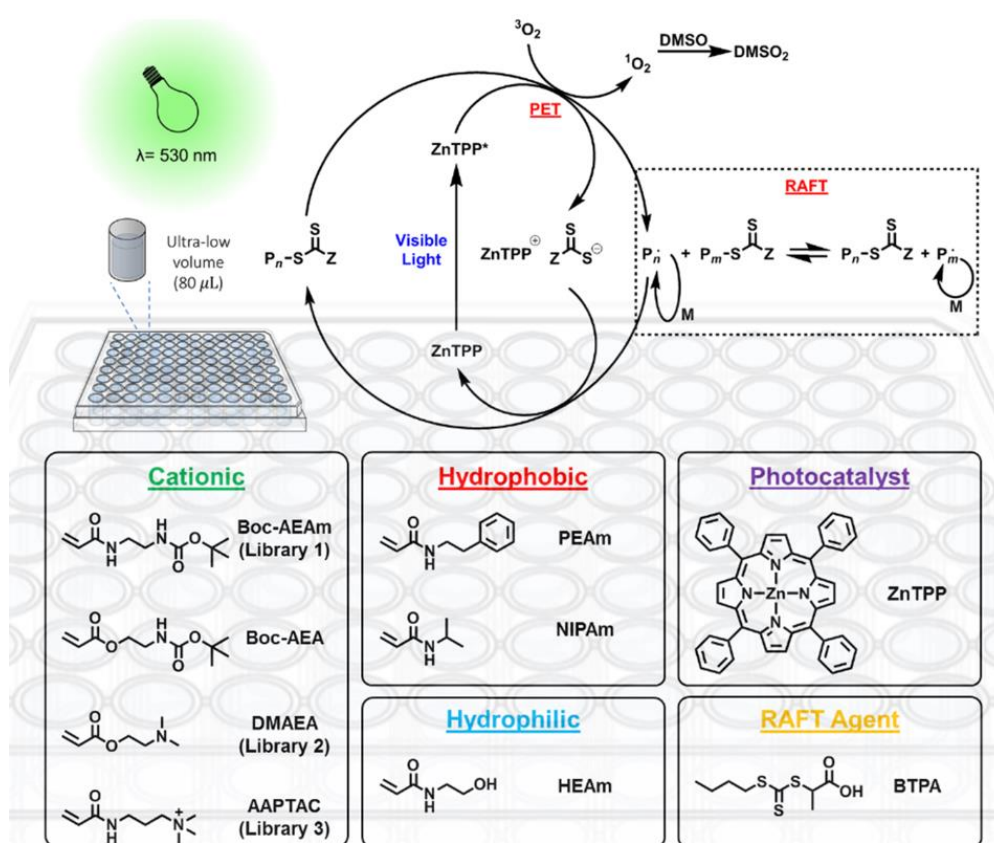
Figure 2.24. The construction of the antimicrobial polymer library through PET-RAFT.[113]

As mentioned above, the derivates of curcumin could co-administrate with ciprofloxacin against gram-positive bacteria. Moreover, the synergy between curcumin and different antibiotics against *M. abscessus* such as amikacin, clarithromycin, ciprofloxacin, and linezolid are shown in **Table 2.3**.[114]

Table 2.3. The synergy and FICI between antibiotics and curcumin against *M. abscessus*.[102]

| Antibiotics | MIC (μg mL^{-1}) | | | | FICI |
| | Individual | | In Combination | | |
	Curcumin	Antibiotics	Curcumin	Antibiotics	
Amikacin	128	32	0.125	0.0625	0.19
Ciprofloxacin	128	8	0.25	0.125	0.38

Clarithromycin	128	128	0.125	0.125	0.25
Linezolid	128	32	0.25	0.0078	0.26

Additionally, numerous other studies performed regarding the synergy between antibiotics against mycobacterium. The rifabutin had synergy with tigecycline and clarithromycin.[115] The combination of teicoplanin and tigecycline showed synergy.[116] These combinations had no direct relationship with AMPs or mimics, but could contribute to further studies, such as the ternary synergetic system with antimicrobial polymers, AMPs, and conventional antibiotics against mycobacterium.

Chapter 3

Synergetic Tri-Systems based on Antimicrobial Polymers against Gram-Negative Bacteria

3.1 Introduction

Previously, the research group at UNSW described the use of a statistical ternary antimicrobial copolymer (**P**) that specifically synergizes with either doxycycline (**D**) or the AMP colistin methanesulfonate (**C**) in a two-component system against Gram-negative bacteria *Pseudomonas aeruginosa*.[28] The observed synergy between **P** and **C** was especially interesting considering that both macromolecules proceed via the membrane disruption mechanism. Even though Typas and coworkers[84] have found in a recent seminal study that synergy is more common for antimicrobial agents that share the same mechanism. It was least expected that two different membrane-disrupting type antimicrobials are more efficient in combination than individually. This then raised an interesting possibility: could the mixture of **P** and **C** be considered as a new, more potent 'single' entity that can be applied further in combination therapy with other antibiotics?

Therefore, it is the aim of this chapter to explore this possibility via the development of a new combination therapy tri-system against *P. aeruginosa* that include two different membrane-disrupting type antimicrobial agents, **P** and **C**, in conjunction with the antibiotic **D**, rifampicin (**R**) or azithromycin (**A**) (**Figure 3.1**). The antibiotics were chosen for specific reasons. Firstly, **D** showed good synergy with **P** in a binary system based on the earlier work and thus represents the system with the best chance of success.[28] On the other hand, **R** and **A** are not typically used on Gram-negative bacteria and hence serves as excellent models to determine the limits of the **P** and **C** combination. To the

best of knowledge, this is the first study that describes the use of an antimicrobial polymer in a combination therapy tri-system.
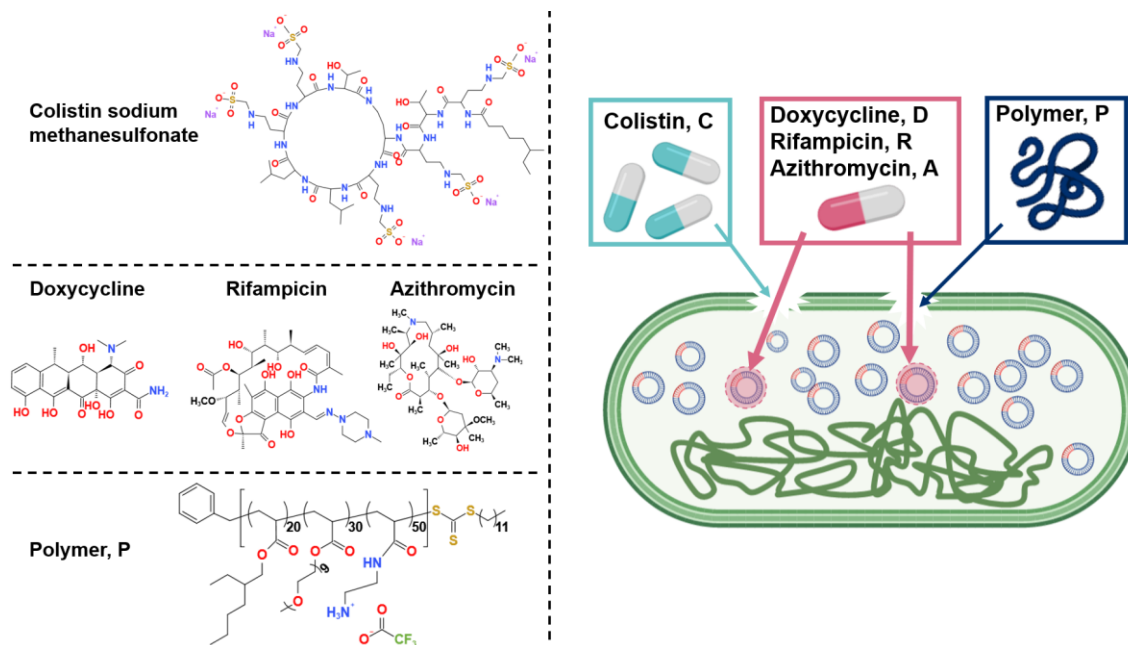
Figure 3.1. Chemical structures of all compounds investigated in this chapter (left panel) and the schematic (right panel) showing the possible mechanism of action involving two membrane disrupting antimicrobial agents (antimicrobial polymer **P** and colistin methanesulfonate **C**) in concert with the antibiotics doxycycline **D**, rifampicin **R** or azithromycin **A**.

3.2 Materials and Methods

Materials. The antimicrobial statistical ternary copolymer **P** was made via RAFT polymerization (by taking to near quantitative monomer conversion), followed by removal of Boc groups using trifluoroacetic acid as previously described.[64] Antibiotics azithromycin dihydrate (≥98%), colistin sodium methanesulfonate (11,500 U mg−1), doxycycline hydrochloride (≥96%), and rifampicin (≥97%) were purchased from Sigma-Aldrich and used as received.

Bacteria Strains. Various strains of the Gram-negative bacteria *Pseudomonas aeruginosa* were used in this chapter, specifically PAO1, and a MDR strain PA32, which were isolated from patients with microbial keratitis.[117, 118]

Minimum Inhibitory Concentration (MIC). The MICs of synthetic polymer and antibiotics were determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[119] Briefly, a single bacterial colony was cultured in 10 mL of Mueller-Hinton Broth (MHB) at 37 °C with 200 rpm shaking overnight. Subsequently, a subculture was prepared from the overnight culture by diluting 1:100 in 10 mL MHB and allowed to grow to mid-log phase, then diluted to circa 1×10^6 cells mL^{-1} for the MIC test. A two-fold dilution series (e.g., 256, 128, 64, 32, … μg mL^{-1}) of 100 μL of polymers or antibiotics solution in MHB were added into 96-well microplates (Costar, Corning), followed by the addition of 100 μL of the subculture suspension. The final concentration of bacteria in each well was circa 5×10^5 cells mL^{-1}. After incubating the plate at 37 °C for 20 h, the absorbance at 600 nm was measured using a microtiter plate reader (FLUOstar Omega, BMG Labtech). MIC values were defined as the lowest concentration of sample that showed no visible bacteria growth and inhibited more than 90% bacteria growth. Positive controls without polymer and negative controls without bacteria were included. All assays included duplicates and were repeated in at least three independent experiments.

Checkerboard Assay. The checkerboard assay for tri-system containing antibiotics and polymer was performed in 96-well microplates (Costar, Corning) in MHB.

Concentration gradients between **C** and different antibiotics were prepared in the horizontal and vertical direction in a 9×7 layout, followed by the addition of polymer **P** at a fixed concentration across each well. Bacterial suspensions were prepared in the same manner as for the MIC test above and added to the plate. As an example, each well may contain two types of antibiotics (2×50 µL), polymer **P** (50 µL), and 50 µL of bacterial suspensions. Positive and negative controls, without antimicrobial agent and bacteria respectively, were also included. The plates were incubated at 37 °C for 20 h, and the absorbance at 600 nm was recorded subsequently. The rows and columns were screened for fractional inhibitory concentration index (FICI). FICI values were calculated via the following **Equation 3.1**: [42]

$$FICI = \frac{MIC_1 \ in \ combination}{Individual \ MIC_1} + \frac{MIC_2 \ in \ combination}{Individual \ MIC_2} + \frac{MIC_3 \ in \ combination}{Individual \ MIC_3}$$

Equation 3.1. The equation of fraction inhibitory concentration index for tri-system, $MIC_{1, 2, \ or \ 3}$ represents the MIC of component 1, 2, or 3 in combination therapy

The FICI data was interpreted as follows: ≤ 0.5, synergistic effect; $0.5 < FICI < 1$, adjuvant effect; and ≥ 4, antagonistic. All experiments were repeated in at least three independent experiments.

Killing Study. To evaluate the bactericidal efficiency of the tri-systems, a time-kill study was conducted against *P. aeruginosa* PAO1 in MHB. Bacterial suspensions were prepared in the same manner as with the MIC and checkboard assays. Selected tri-system (CDP tri-system herein due to the better FICI than CRP and CAP tri-system) combinations (at $1 \times MIC$ and $2 \times MIC$) were incubated with bacteria suspension for a

predetermined time (1, 3 and 20 h). The viability of planktonic cells was then determined by a drop plate method where the planktonic cells were serially diluted in sterile PBS and plated onto Luria Bertani agar. After 24 h of incubation at 37 °C, bacteria colonies were counted and colony forming unit (CFU) analysis was performed. All assays included two replicates and were repeated in at least three independent experiments.

Cytotoxicity Assay. The cytotoxicity of polymer **P**, antibiotics, binary, and tri-systems were determined by alamarBlue assay (Thermo Fisher Scientific) on murine embryonic fibroblasts (MEFs) CF-1 (ATCC SCRC-1040), which was kindly provided by the Cell Culture Facility of the Mark Wainwright Analytical Centre at UNSW. Using a cell culture incubator (Eppendorf CellXpert C170i), MEFs were cultured to subconfluency at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) that was supplemented by L-glutamine (2 mmol, Sigma-Aldrich) and fetal bovine serum (10% (v/v), Sigma-Aldrich). For the cytotoxicity assay, MEFs were subcultured twice and diluted to a final concentration of 5×10^4 cells/mL in DMEM. After adding 100 μL of cell suspension to each well of a flat-bottom, black cell-culture-grade 96-well plate, the plate was cultured for 20 h at 37 °C and 5% CO_2 to attach the cells. Then, the supernatant was aspirated, and 50 μL of fresh DMEM was added, followed by another 50 μL of antimicrobials in DMEM. Subsequently, the plate was incubated for 24 h at 37 °C and 5% CO_2. The fluorescence was measured (excitation 550 nm and emission 590 nm) using a microplate reader (CLARIOstar, BMG Labtech) after adding 10 μL of alamarBlue agent in each well and culturing for 4 h at 37 °C and 5% CO_2. The IC_{50} values were defined as

the concentration with less than 50 % cell viability. All assays included duplicates and were repeated in at least three independent experiments.

3.3 Results and Discussion

As anticipated with the use of controlled polymerization techniques[120-125] like RAFT polymerization, **P** has narrow molecular weight distribution as evidenced by a dispersity value of 1.3.[126] It is worth noting that **P** was designed to consist of three key functional groups – cationic primary amines, hydrophobic groups (2-ethylhexyl) and neutral polar side-chains (polyethylene glycol) – to impart antimicrobial activity against Gram-negative bacteria. The absence of any of these functional groups has shown to negatively impact the antimicrobial activity of the polymer.[64] For instance, the absence of hydrophobic groups renders the polymer incapable of disrupting the bacteria cell membrane effectively and thus resulting in no activity, whereas the lack of polyethylene glycol side-chains leads to the formation of polymer-protein complexes in biological media which in turn masks the presentation of cationic and hydrophobic groups to bacteria cells and hinder the antimicrobial activity.[64]

In light of previous studies[58-60, 126-130], this chapter tested the antimicrobial efficacy of tri-systems on Gram-negative *P. aeruginosa*. *P. aeruginosa* exhibits multidrug resistance and virulence remaining one of the highest concern pathogens according to the World Health Organization.[131] For the initial antimicrobial testing, wild type *P. aeruginosa* PAO1 strain was used. To determine the potential synergistic antimicrobial performance of the tri-system, checkerboard assay was used where the minimum inhibitory

concentration (MIC) values of the compounds alone and in combinations were evaluated (**Table 3.1**). The experimental setup for this tri-system checkerboard required the establishment of concentration gradients between **C** (component 1) and the antibiotic of interest (component 2, i.e. **D**, **R** or **A**) in a 96-well plate, followed by the addition of **P** (component 3) at a fix concentration across the gradients. The bacteria solution was then added last to the wells. The final concentrations used for **P** were set at 0, 1/16 and 1/8 of the individual MIC of **P**. The fractional inhibitory concentration index, FICI (as shown in **Equation 3.1** above), is a common parameter for determining whether a combination at a specific set of concentration will yield a synergistic effect. If a particular combination of concentrations showed synergy, a FICI value of ≤ 0.5 is obtained whereas for an adjuvant effect, the FICI is greater than 0.5 and less than 1.

Table 3.1. MIC and FICI results of the adjuvant and synergistic points for the different tri-systems against *P. aeruginosa* PAO1 through the checkerboard assay

	MIC (μg mL^{-1})						
	Individual			In tri-system			
	C	D	P	C	D	P	FICI
				2	8	0	0.56
				4	4	0	0.38
				8	2	0	0.38
				16	1	0	0.56
				1	8	4	0.59
				2	4	4	0.38
CDP	32	16	64	4	2	4	0.31
				16	0	4	0.56
				0	8	8	0.63
				0.5	4	8	0.39
				2	2	8	0.31
				4	1	8	0.31

	C	R	P		C	R	P	FICI
					8	0	8	0.38
CRP	16	32	64		4	16	0	0.75
					8	2	0	0.56
					2	16	4	0.69
					4	4	4	0.44
					8	0	4	0.56
					1	8	8	0.44
					2	4	8	0.38
					4	1	8	0.41
					8	0	8	0.63

	C	A	P		C	A	P	FICI
					4	64	0	0.75
					8	4	0	0.53
					2	64	4	0.69
					4	8	4	0.38
CAP	16	128	64		8	4	4	0.59
					1	64	8	0.69
					2	32	8	0.50
					4	4	8	0.41
					8	0	8	0.63

Upon inspection of the checkerboard plots for the **CDP** tri-system, various synergy points were detected as verified by their FICI values (**Figure 3.2** and **Table 3.1**). The coadministration of these agents against *P. aeruginosa* PAO1 resulted in up to 4- to 8-fold decrease with respect to their individual MIC values. For the **CRP** and **CAP** systems, synergy points were also observed albeit lesser and with higher FICI values. This indicated that the combination of **C** and **P** with **D** yield the most potent activity against PAO1 than the other two antibiotics. Furthermore, it was noticed that **CRP** and **CAP** systems only produced an adjuvant effect when $[\mathbf{P}] = 0 \ \mu g \ mL^{-1}$, in contrast to the **CDP** system that showed synergy even in the absence of **P**. This showed that **R** and **A** are not as strong as **D** in working concertedly with **C** against *P. aeruginosa*. In spite of this, the

addition of **P** resulted in improved synergistic interactions in the **CRP** and **CAP** systems, thus suggesting the benefit of concurrently using two different membrane disrupting antimicrobial agents to potentiate the activity of other antibiotics. For instance, the MIC of **A** alone was 128 µg mL^{-1} but decreased to 4-8 µg mL^{-1} in the tri-system. While it is difficult to pinpoint the exact antimicrobial mechanisms of the tri-systems, it is hypothesized that the combination of **P** and **C** would weaken the cell membrane of the bacteria to a greater extent than on their own to enable antibiotics such as **D**, **R** and **A** to act on the intracellular targets more effectively.
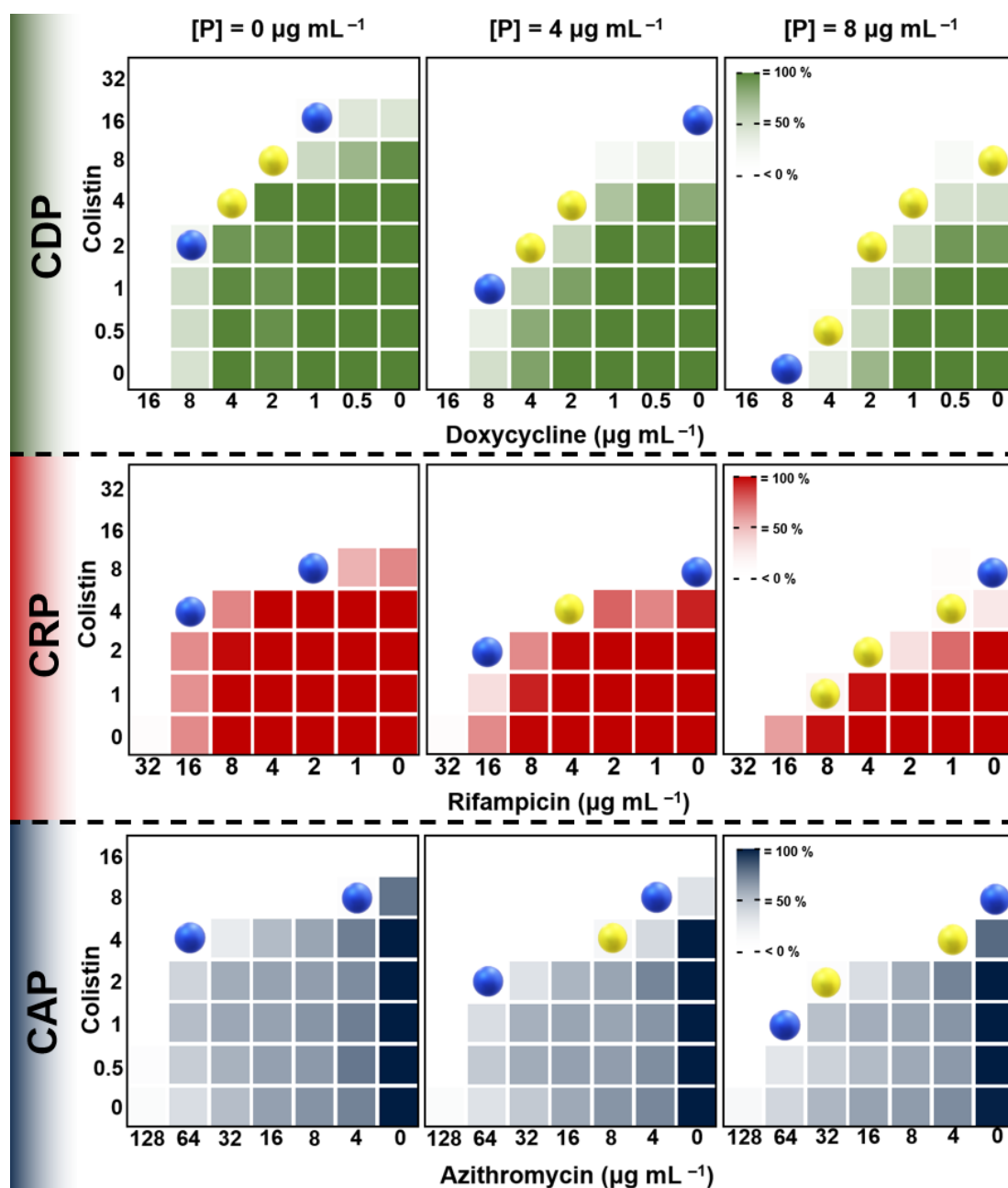
Figure 3.2. Checkerboard microdilution assay of the tri-systems **CDP** (green), **CRP** (red), and **CAP** (navy blue) against *P. aeruginosa* PAO1. The bacterial growth, as quantified by the average optical density measured at 600 nm, is illustrated as a linear gradient from white to the respective colors where darker shades represent higher percentage of bacteria (i.e., less growth inhibition). Yellow and blue bullets indicate concentration coordinates showing synergistic and adjuvant interactions, respectively. The data are based on at least two biological replicates.

The synergetic interactions of the tri-systems were also evaluated against a MDR *P. aeruginosa* strain PA32, which is an isolate from microbial keratitis.[117, 118] A similar outcome was observed for the **CDP** system against PA32 compared to PAO1 where multiple synergy points occurred (**Figure 3.3** and **Table 3.2**). However, as expected, the MIC of **D** against PA32 was considerably higher than it was for PAO1 (512 vs 16 µg mL^{-1}) because of the MDR nature of PA32. With the addition of **P** and **C** to **D**, FICI value as low as 0.25 was attained, thus confirming the cooperative interactions between the 3 compounds even against a MDR strain. Based on the pattern of the checkerboard plots for the **CRP** system, it was evident that weaker interaction was observed for PA32 than PAO1 when **P** was at 1/8 of its own MIC. Specifically, no apparent synergy or adjuvant interactions were observed between **C** and **R** at [**P**] = 16 µg mL^{-1} although **P** was clearly able to have synergistic and adjuvant interactions with **R** and **C**, respectively. The reason for this diminished interaction between **C** and **R** in the presence of higher concentration of **P** (and not lower) is not entirely clear though the results suggests that when enough **P** is present, **R** will preferentially interact with **P** over **C** almost exclusively, bearing in mind that **C** and **R** are not strongly interacting with each other even in the absence of **P**. Meanwhile, the interactions for the **CAP** system against PA32 was similar to that for PAO1. As **A** is an antibiotic that is commonly used to treat Gram-positive infections rather than Gram-negative, the MIC of **A** was high (for an antibiotic) against both PAO1 and PA32 (128 µg mL^{-1}). Nonetheless, the addition of **P** and **C** allowed **A** to demonstrate susceptibility towards *P. aeruginosa* that is otherwise not very effective as a standalone

antibiotic. <mark>The stronger synergetic interaction of **CDP** (lower FICI) than **CRP** and **CAP** against both PAO1 and PA32 is attributed to the sufficient binary synergy of the two commercial antibiotics inside which was observed under the 0 concentration of **P**.</mark>
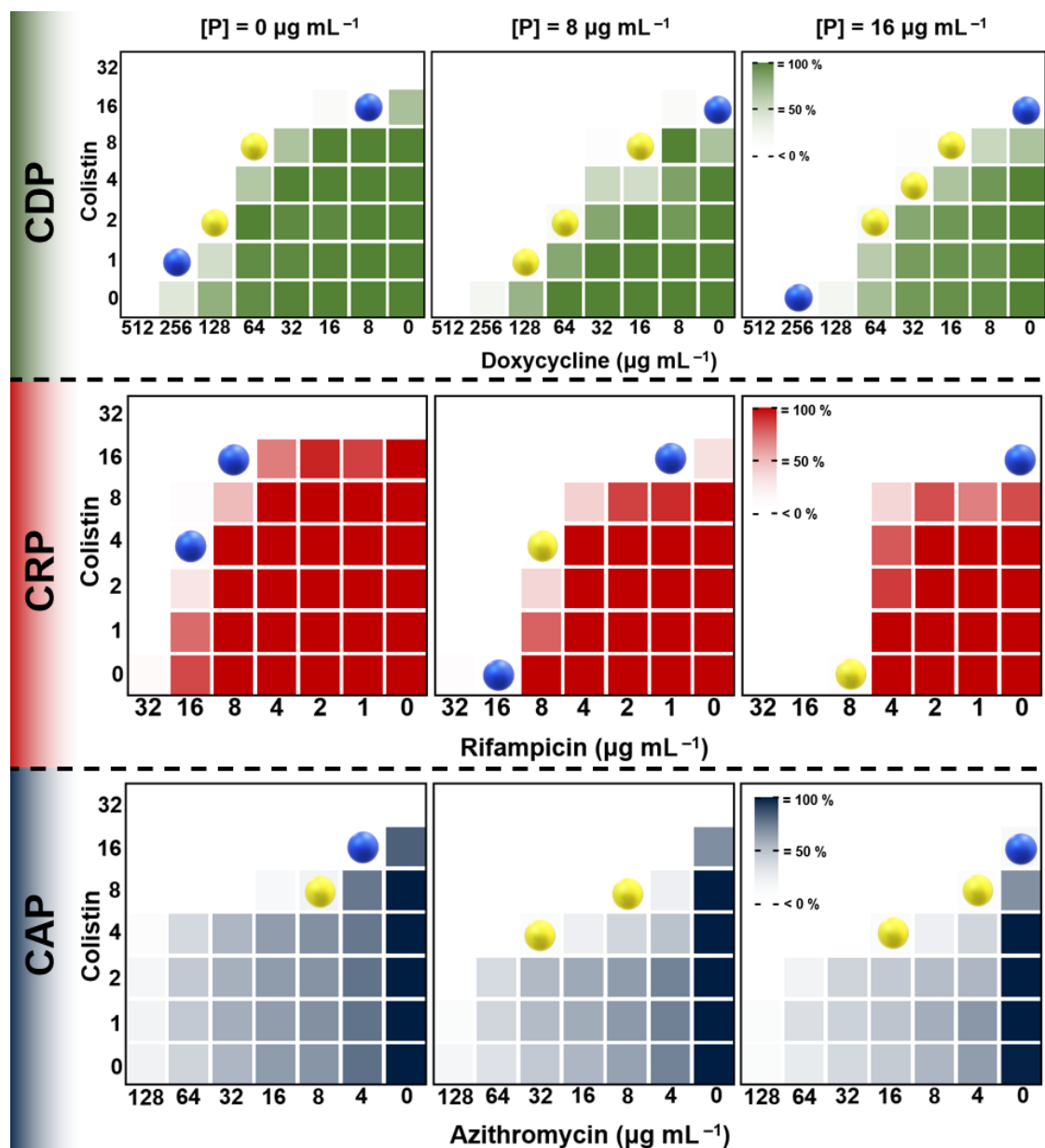
Figure 3.3. Checkerboard microdilution assay of the tri-systems **CDP** (green), **CRP** (red), and **CAP** (navy blue) against MDR *P. aeruginosa* PA32. The bacterial growth, as quantified by the average optical density measured at 600 nm, is illustrated as a linear gradient from white to the respective colors where darker shades represent higher percentage of bacteria (i.e., less growth inhibition). Yellow and blue bullets

49

indicate concentration coordinates showing synergistic and adjuvant interactions, respectively. The data are based on at least two biological replicates.

Table 3.2. MIC and FICI results of the checkerboard assay for the different tri-systems against MDR *P. aeruginosa* PA32

	MIC (μg mL^{-1})						
	Individual			In tri-system			
	C	D	P	C	D	P	FICI
				1	256	0	0.53
				2	128	0	0.31
				8	64	0	0.38
				16	8	0	0.52
				1	128	8	0.34
				2	64	8	0.25
CDP	32	512	128	8	16	8	0.34
				16	0	8	0.56
				0	256	16	0.63
				2	64	16	0.31
				4	32	16	0.31
				8	16	16	0.41
				16	0	16	0.63
	C	R	P	C	R	P	
				4	16	0	0.63
				16	8	0	0.75
				0	16	8	0.56
CRP	32	32	128	4	8	8	0.44
				16	1	8	0.59
				0	8	16	0.38
				16	0	16	0.63
	C	A	P	C	A	P	
				8	8	0	0.31
				16	4	0	0.53
				4	32	8	0.44
CAP	32	128	128	8	8	8	0.38
				4	16	16	0.38
				8	4	16	0.41
				16	0	16	0.63

The checkerboard assays indicated the bacteriostatic activity of the tri-systems. To determine the bactericidal activity, a time-kill assay was subsequently performed where colony forming unit (CFU) analysis was used to ascertain the bactericidal effect of the tri-systems on planktonic *P. aeruginosa* PAO1 cells at 1, 3 and 20 h time intervals (**Figure 3.4**). The concentration selected for each tri-system was based on their lowest FICI value. For example, for the **CDP** system, the concentrations of the compounds in the combination at $1 \times$ MIC were 4, 2 and 4 μg mL^{-1}, respectively. The initial bacteria loading was ca. 5×10^5 cells mL^{-1}. Minimal reduction in CFU was observed at $1 \times$ MIC for all three tri-systems after 1 and 3 h of incubation, but showed ca. 3.3-log$_{10}$ reduction in CFU after 20 h compared to the negative control. This was not unexpected given that higher concentrations than those used for growth inhibition are usually required to exert good bactericidal effect. At $2 \times$ MIC, there was a clear difference in bactericidal activity between the three tri-systems. The **CDP** and **CAP** systems did not yield any appreciable reduction in CFU after 1 h but the **CRP** system resulted in 3.5-log$_{10}$ reduction in CFU with respect to the control. In fact, no colonies were actually detected, and it is worth noting that the lowest detection limit was conservatively set at 2-log$_{10}$ CFU mL^{-1} even though the system may have completely inactivated all the bacteria. The same observation was made for the **CRP** system at 3 and 20 h time points. At the 3 h mark, the **CDP** system showed potent bactericidal activity and produced no detectable colonies, which remained likewise after 20 h of incubation. The **CAP** system was the least bactericidal though still resulted in 5.9-log$_{10}$ reduction in CFU after 20 h compared to the control sample. The

time-kill experiments provided some interesting insights. Coincident or not, both **D** and **A**, which act by targeting the ribosomal units to inhibit protein synthesis, happened to exhibit more inferior bactericidal activity than **R**, which proceeds via inhibition of bacterial RNA polymerase. This may imply that the combination of **P** and **C** with an antibiotic that disrupts DNA transcription will yield high bactericidal activity against Gram-negative bacteria. In addition, the better bacteriostatic activity and the stronger synergetic interaction of **CDP** system were speculated to exhibit a positive correlation. However, this potential relationship could not be extended to the relevance between bactericidal effect and synergetic degree since the OD_{600} in checkerboard assays only reflected the inhibition of bacterial growth.
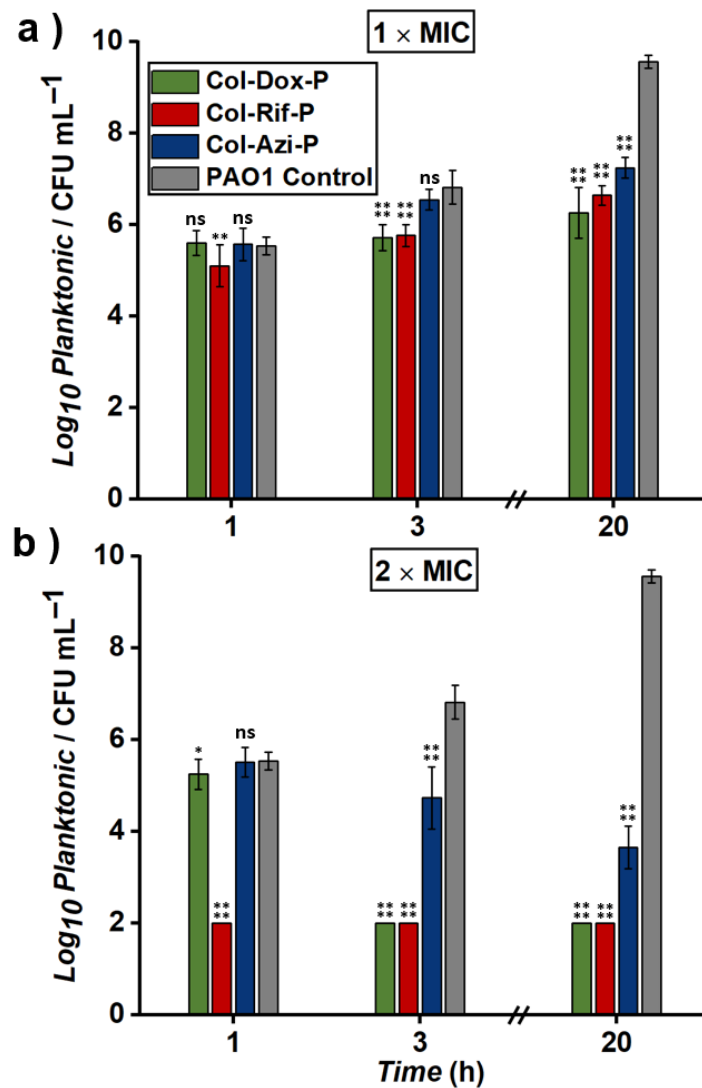
Figure 3.4. Time-kill assay of selected **CDP**, **CRP**, and **CAP** tri-systems against planktonic *P. aeruginosa* PAO1 cells as determined via CFU analysis. The concentrations of each compound in the combinations at 1 × MIC are as followed: **CDP** – 4, 2, 4 μg mL^{-1}; **CRP** – 2, 4, 8 μg mL^{-1}; **CAP** – 4, 8, 4 μg mL^{-1}. Data are representative of at least three independent experiments. Two-tailed student's t-test; asterisks indicated a statistically significant difference of each tri-system vs bacterial control group (*$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$; ****$p < 0.00001$; ns, nonsignificant ($p > 0.01$)).

Next, this chapter evaluated the in vitro biocompatibility of a tri-system with a model mammalian cell line such as murine embryonic fibroblasts (MEFs). The **CDP** system was chosen using the combination that showed the best FICI value (like in the bactericidal study above). For this, the metabolic activity of MEFs after 24 h incubation with

individual, different binary and ternary combinations of **C**, **D** and **P** was evaluated by alamarBlue assay, and visualized as equilateral triangular heatmaps in **Figure 3.5**. In essence, the biocompatibility was purely dependent on **P**. Even when all three components were present at once, thus making the total concentration of compounds in **CDP** as high as 160 μg mL^{-1} (i.e., 64 + 32 + 64 μg mL^{-1}), the metabolic activity of the combination was comparable to that of **P** alone at 64 μg mL^{-1} (**Figure 3.5c**). This crucially proves that despite the synergistic potentiation in antimicrobial activity, as evidenced above, this effect is only reserved for bacteria and does not translate to equitoxicity for mammalian cells. This point has never been explicitly demonstrated until herein. The IC$_{50}$ for **P** and **CDP** were ca. 128 and 320 μg mL^{-1}, respectively. Taking into account the MIC values of **P** and **CDP** against PAO1 were at 64 and 10 μg mL^{-1}, the corresponding TI values were 2 and 32 (calculated through **Equation 1.1**), respectively. This represents a massive improvement in TI from the perspective of **P**. It is worth noting that the binary system **DP** only has a TI value of 12 (based on IC$_{50}$ and MIC values of 192 and 16 μg mL^{-1}, respectively), which is still better than **P** alone but not as superior compared to the **CDP** tri-system.
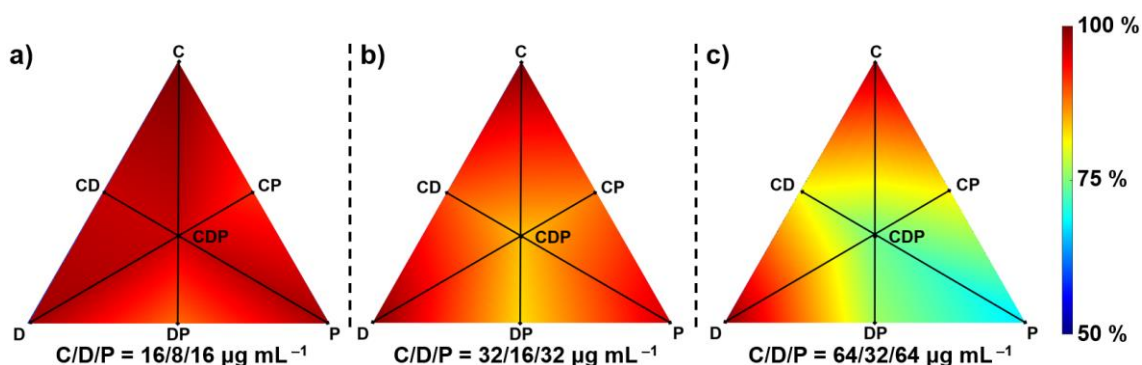
Figure 3.5. The mammalian cell biocompatibility study of the **CDP** tri-system. The cell viability, as quantified by the average fluorescent intensity measured at excitation and emission wavelengths of 550 and 590 nm respectively, is illustrated as an equilateral triangular heatmap generated using MATLAB. Each figure with different tri-system concentration is constructed by 6 smaller right triangles inside that each represents 6 individual Cartesian coordinate system. The metabolic activity of sole components, binary, and ternary combinations was indicated at vertex angles, midpoints of sides, and centroids, respectively. In each triangle coordinate system, the axis reflected the increasing concentration of one element in the tri-system. The relationship between the increasing concentration of each element and the change in metabolic activity was assumed to be linear. The concentration of each compound is listed below the heatmaps. The data are based on at least three biological replicates.

While there may be concerns regarding the feasibility of having all 3 components at the infection site in vivo, recent studies on binary combination systems involving antimicrobial polymers have shown the successful translation of in vitro antimicrobial efficacies to in vivo animal models (via intraperitoneal and tail vein injections in mouse).[11, 24] This offers hope that the tri-systems demonstrated here may achieve similar potency in vivo as the in vitro results. However, to ensure the efficient delivery of all components to the target site, 'smart' drug delivery systems may be employed where the antimicrobial agents could be encapsulated and released at the infection site in response to a stimuli/trigger.[132] This could be an attractive avenue to increase the feasibility of this tri-system strategy for clinical applications. It is envisaged that the tri-system will most likely be more suitable for topical (local) or intravenous than oral administration routes in humans because of the potential degradation of antimicrobial agents in the gastrointestinal tract.

3.4 Conclusion

In summary, this chapter described the development of novel combination therapy tri-systems that contain two membrane-disrupting type antimicrobial agents, namely the synthetic cationic antimicrobial ternary copolymer **P** and the AMP colistin methanesulfonate **C**, in combination with one of the antibiotics doxycycline **D**, rifampicin **R**, or azithromycin **A**. All three tri-systems exhibited synergy characteristic although the **CDP** system was the best, as confirmed by the higher number of synergy points and lower FICI values against Gram-negative bacteria *Pseudomonas aeruginosa* including a MDR strain. Besides being bacteriostatic, the tri-systems were also bactericidal at $2 \times$ MIC, where the **CRP** combination showed the most potent activity. In addition, in vitro biocompatibility experiments with mouse embryonic fibroblasts importantly revealed that the tri-system combination (exemplified using the **CDP** system) did not result in higher toxicity to the mammalian cells despite showing synergistic potentiation in antimicrobial activity. This crucially led to the substantial improvement of the therapeutic/selectivity index, which increased from 2 for **P** alone to 32 for the **CDP** tri-system. Furthermore, the further study of the nano-based drug delivery for these ternary combinations would improve the feasibility of administration.[133] This study thus provides several important insights including the ability to improve therapeutic index using combination therapy and the benefit of using two different membrane-disrupting antimicrobial agents to enhance the activity of antibiotics that are otherwise not effective when used alone.

Chapter 4

Adjuvant Binary Systems based on Antimicrobial Polymers against Mycobacterium

4.1 Introduction

As pointed out in Chapter 3 above, great progress has been made for antimicrobial polymers in terms of potency against Gram-negative and Gram-positive bacteria. In fact, the majority of the literature focused on these targets. Mycobacteria, another family of pathogen worth investigating, fundamentally important for anti-tuberculosis therapy, has been largely overlooked. Only few reports exist regarding the application of antimicrobial polymers against mycobacteria. For example, Gibson, Fulham and co-workers[132] demonstrated the ability of tertiary amine-functionalized homopolymers to selectively target *Mycobacterium smegmatis* over Gram-negative bacteria. In addition, a study by Bajaj, Srivastava and co-workers[134] recently showed that polyamides with quaternary ammonium side-chains could target intracellular mycobacteria.

Fueled by the lack of strategies in literature against mycobacteria and considering the benefits of combination therapy, this chapter thus reveals for the first time the development of a two-component combination therapy platform involving antimicrobial polymers and antibiotics against mycobacteria. Specifically, linear homopolymers containing pendant quaternary ammonium groups (**P2** and **P2-40**, poly-AAPTAC ((3-Acrylamidopropyl) trimethylammonium chloride)) and random copolymers with the same type of amine functionality and hydrophobic phenyl moieties (**P2b-40**, poly-AAPTAC-phenylethyl acrylamide) (**Figure 4.1**) were screened in combination with different classes of antibiotics (**Figure 4.2**) via checkerboard assays against *M. smegmatis*.

P2: m=100

P2-40: m=40

- -

P2b-40: m=20, n=20
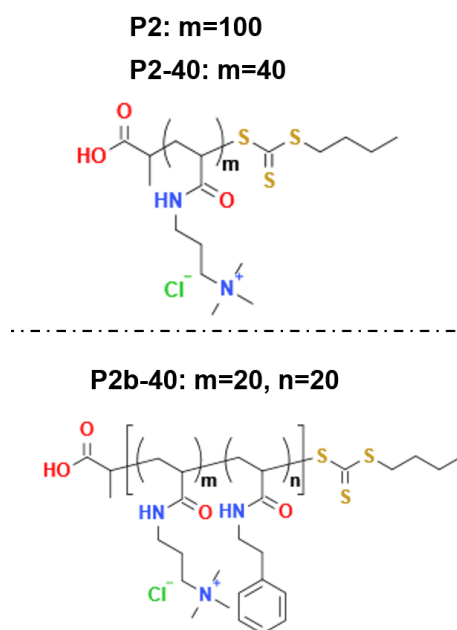
Figure 4.1. The chemical structures of the antimicrobial polymers used in this <mark>chapter</mark>.
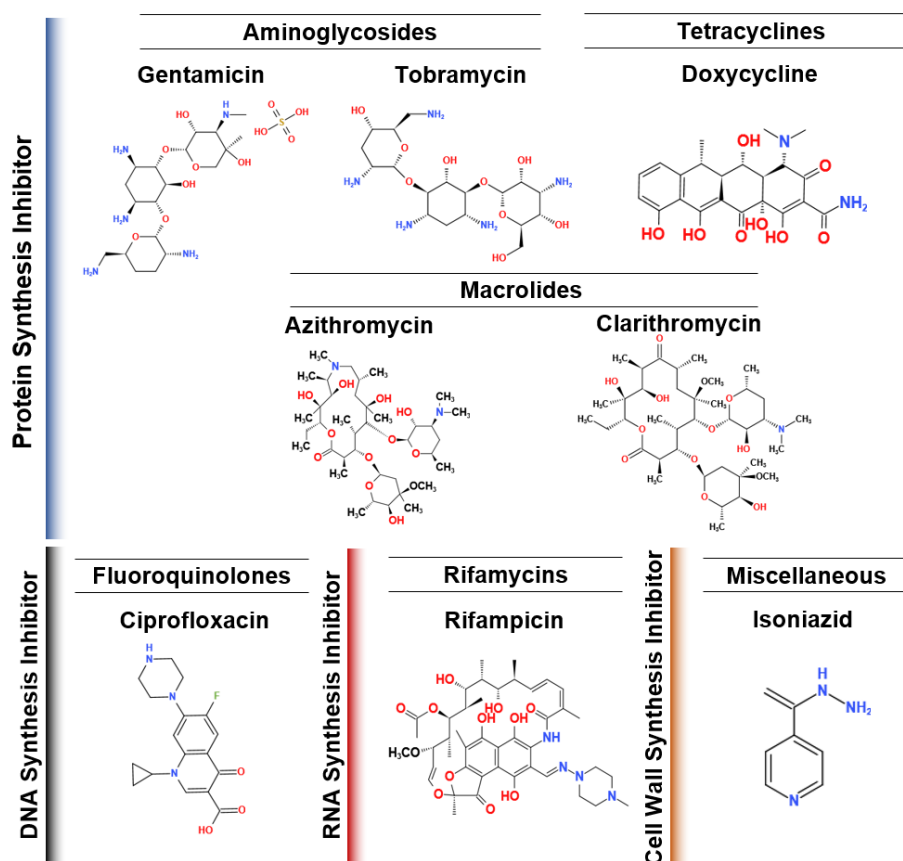
Figure 4.2. The chemical structures of the antibiotics used in this <mark>chapter</mark>.

4.2 Materials and Methods

Materials. The antimicrobial polymers against Gram-positive pathogens were from an earlier study and were used as they are.[113, 126] The monomers (AAPTAC and 2-phenyethyl acrylamide), the RAFT agent (BTPA), the photocatalyst (ZnTPP), the antibiotics (azithromycin dihydrate (≥98%), ciprofloxacin hydrochloride (pharmaceutical primary standard), clarithromycin (pharmaceutical secondary standard), doxycycline hydrochloride (≥96%), gentamicin sulfate (pharmaceutical secondary standard), isoniazid (≥99%), rifampicin (≥97%), tobramycin (pharmaceutical secondary standard)) were purchased from Sigma-Aldrich.

Bacteria Strain. The tested strain herein was *M. smegmatis* ATCC 70084 which was kindly provided by the Centenary Institute (Sydney).

Minimum Inhibitory Concentration (MIC). The MICs of synthetic polymer and antibiotics were determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[119] Briefly, a single bacterial colony was cultured in 15 mL of Middlebrook 7H9 media supplemented with 0.5 % glycerol and 0.05 % Tween-80 at 37 °C with 200 rpm shaking for 72 h. Subsequently, this culture was diluted directly to the appropriate concentration for the MIC test in 7H9 media. A twofold dilution series of 100 μL of polymers or antibiotics solution in 7H9 were added into 96-well microplates (Costar, Corning), followed by the addition of 100 μL of the culture suspension. The final concentration of bacteria in each well was ca. 5×10^5 cells mL^{-1}. After incubating the plate at 37 °C for 72 h, the absorbance at 600 nm was measured using a microtiter plate reader (FLUOstar Omega, BMG Labtech). MIC values were defined as

the lowest concentration of sample that showed no visible bacteria growth and inhibited more than 90% bacteria growth. Positive controls without polymer and negative controls without bacteria were included. All assays included two replicates and were repeated in at least three independent experiments.

Checkerboard Assay. The checkerboard assay of antibiotics and polymers for this chapter was performed in 96-well microplates (Costar, Corning) in 7H9. Concentration gradients between different polymers and antibiotics were prepared in the horizontal and vertical direction in a 9×7 layout. Bacterial suspensions were prepared in the same manner as for the MIC test above and added to the plate. Positive and negative controls, without antimicrobial agent and bacteria respectively, were also included. The plates were incubated at 37 °C for 72 h, and the absorbance at 600 nm was recorded subsequently. The rows and columns were screened for fractional inhibitory concentration index (FICI). FICI values were calculated via the following **Equation 4.1**: [42]

$$FICI = \frac{MIC_1 \text{ in combination}}{Individual \ MIC_1} + \frac{MIC_2 \text{ in combination}}{Individual \ MIC_2}$$

Equation 4.1. The equation of fraction inhibitory concentration index for binary system

The FICI data was interpreted as follows: ≤ 0.5, synergistic effect; $0.5 < FICI < 1$, adjuvant effect; and ≥ 4, antagonistic. All experiments were repeated in at least three independent experiments.

Cytotoxicity Assay. The cytotoxicity of polymers (**P2**, **P2-40**, **P2b-40**), gentamicin, and polymer-antibiotic binary systems thereof were determined by alamarBlue assay (Thermo Fisher Scientific) on murine embryonic fibroblasts (MEFs) CF-1 (ATCC SCRC-

1040), which was kindly provided by the Cell Culture Facility of the Mark Wainwright Analytical Centre at UNSW. Using a cell culture incubator (Eppendorf CellXpert C170i), MEFs were cultured to subconfluency at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) that was supplemented by L-glutamine (2 mmol, Sigma-Aldrich) and fetal bovine serum (10% (v/v), Sigma-Aldrich). For the cytotoxicity assay, MEFs were subcultured twice and diluted to a final concentration of 5×10^4 cells/mL in DMEM. After adding 100 μL of cell suspension to each well of a flat-bottom, black cell-culture-grade 96-well plate, the plate was cultured for 20 h at 37 °C and 5% CO_2 to attach the cells. Then, the supernatant was aspirated, and 50 μL of fresh DMEM was added, followed by another 50 μL of antimicrobials in DMEM. Subsequently, the plate was incubated for 24 h at 37 °C and 5% CO_2. The fluorescence was measured (excitation 550 nm and emission 590 nm) using a microplate reader (CLARIOstar, BMG Labtech) after adding 10 μL of alamarBlue agent in each well and culturing for 4 h at 37 °C and 5% CO_2. The IC_{50} values were subsequently estimated. All assays included two replicates and were repeated in at least three independent experiments.

4.3 Results and Discussion

The antimicrobial polymers were made via photoinduced electron transfer-RAFT polymerization and were taken to full monomer conversion, thereby allowing the formed polymers to be used directly for biological testing and avoid potentially cumbersome purification steps. Because of different bacterial membrane structures, distinct polymer construction was investigated previously when targeting diverse bacterial families.[60, 64,

62

[113] Based on earlier studies, the homo- (**P2** and **P2-40**) and random copolymers (**P2b-40**) demonstrated the excellent activity against *M. smegmatis* and hence were chosen for screening in this chapter. Furthermore, the homo- and random copolymers will enable for good comparison study to determine the influence of chain length (100 vs 40 monomer repeat units) and hydrophobicity (0 vs 50 mol% hydrophobic phenyl groups) in antimicrobial polymers during combination therapy with antibiotics against mycobacteria.

Initially, the homopolymer **P2** with ca. 100 repeat units of quaternary ammonium side-chains were used to screen with different antibiotics via checkerboard assay to identify the presence of synergistic or adjuvant concentration combinations (**Figure 4.3**). The checkerboard plots in **Figure 4.3** revealed that **P2** has adjuvant interactions with gentamicin and tobramycin as well as rifampicin, though the interactions with aminoglycosides (gentamicin or tobramycin) were slightly stronger than that with the rifamycin antibiotic, as verified by their FICI values (**Table 4.1**). Unfortunately, no synergy points were observed among all the screened antibiotics that were known to exhibit antimycobacterial activity.

Table 4.1. MIC and FICI values of **P2** and antibiotics during mono and combination therapies against *M. Smegmatis[a]*

| Antibiotics | Polymers | MIC (μg mL^{-1}) | | | | FICI |
| | | Individual | | In Combination | | |
		Antibiotics	Polymer	Antibiotics	Polymer	
Azithromycin	P2	4	32	–	–	NI
Ciprofloxacin	P2	0.25	32	–	–	NI

Clarithromycin	P2	0.5	32	–	–	NI
Doxycycline	P2	0.5	32	–	–	NI
Isoniazid	P2	16	32	–	–	NI
Gentamicin	P2	4	32	2	4	0.63
				0.5	16	0.63
Tobramycin	P2	2	32	1	8	0.75
				0.5	32	0.63
Rifampicin	P2	2	32	1	16	1

[a] –, none; NI, No interaction.
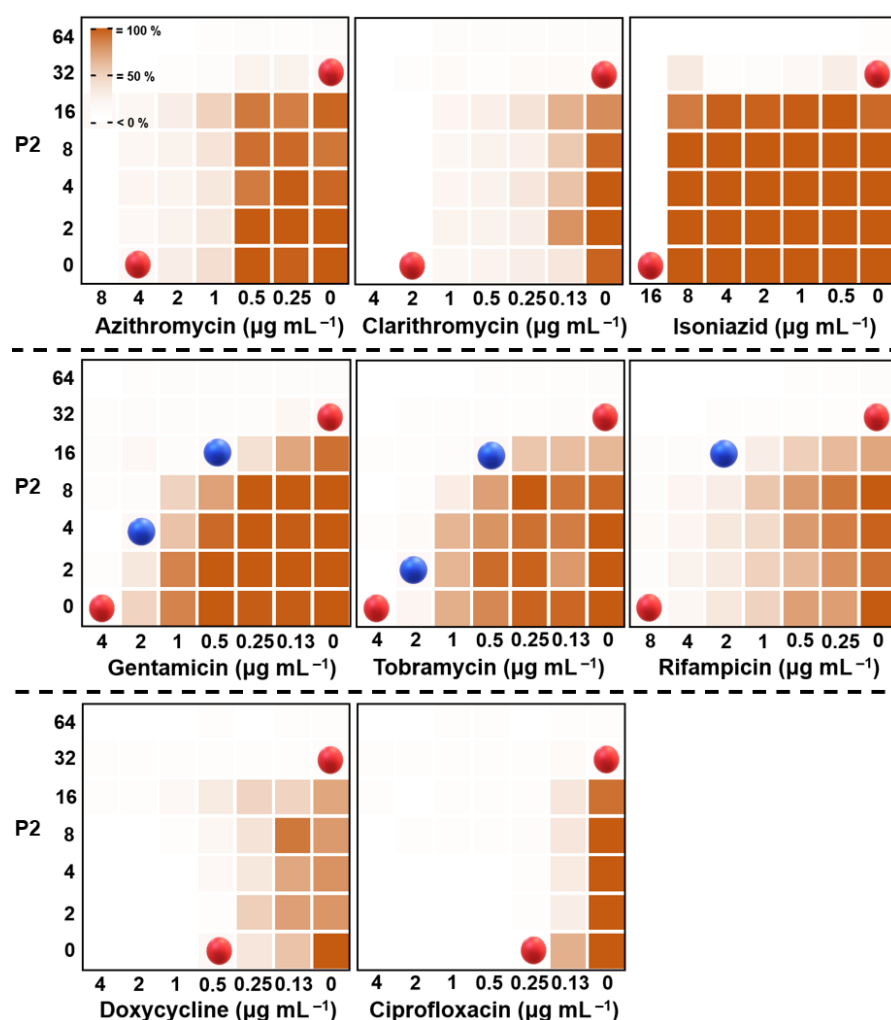
Figure 4.3. The checkerboard assays of **P2** and different antibiotics against *M. smegmatis*. The bacterial growth, as quantified by the average optical density measured at 600 nm, is illustrated as a linear gradient from white to brown where darker shades represent higher percentage of bacteria (i.e., less growth

inhibition). Red and blue bullets indicate concentration coordinates showing individual MIC values and adjuvant concentrations, respectively. The data are based on at least two biological replicates.

After the initial screening, a more focused approach was adopted where the shorter chain length polymers were investigated for their interactions with gentamicin and tobramycin (**Figure 4.4** and **Table 4.2**). Similar to the results obtained for **P2**, the checkerboard plots in **Figure 4.4** showed that both **P2-40** and **P2b-40** produced only adjuvant interactions and comparable FICI values, thus suggesting that lower chain length and the inclusion of hydrophobic moieties have negligible effect on the outcome of the combination therapy with aminoglycosides.
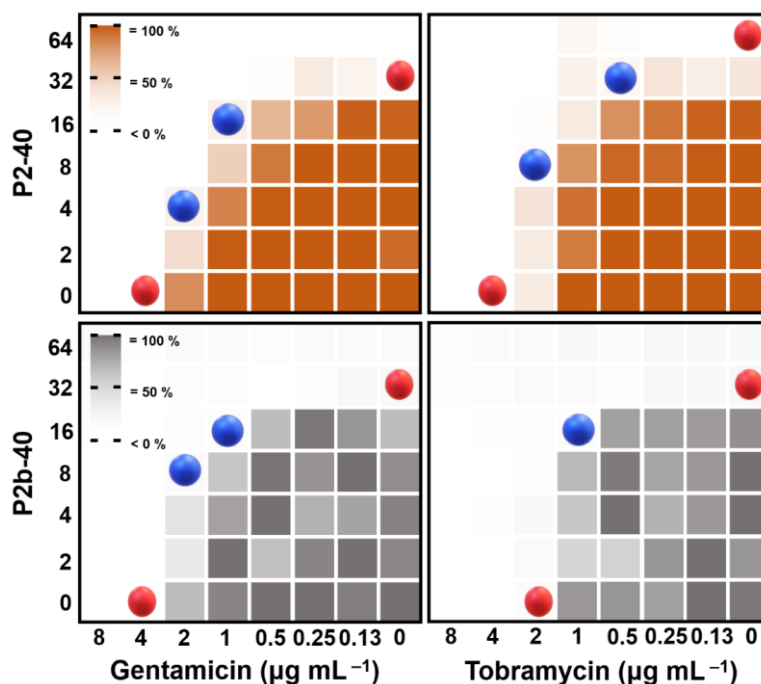
Figure 4.4. The checkerboard assays of **P2-40** (orange) and **P2b-40** (grey) with gentamicin and tobramycin against *M. smegmatis*. The bacterial growth, as quantified by the average optical density measured at 600 nm, is illustrated as a linear gradient from white to the respective colors where darker shades represent higher percentage of bacteria (i.e., less growth inhibition). Red and blue bullets indicate

concentration coordinates showing individual MIC values and adjuvant concentrations, respectively. The data are based on at least two biological replicates.

Table 4.2. MIC and FICI values of shorter chain length polymers **P2-40** and **P2b-40** and aminoglycosides during mono and combination therapies against *M. Smegmatis*

| Antibiotics | Polymers | MIC (µg mL $^{-1}$) | | | | FICI |
| | | Individual | | In Combination | | |
		Antibiotics	Polymer	Antibiotics	Polymer	
Gentamicin	P2-40	4	32	2	4	0.63
				1	16	0.75
Tobramycin	P2-40	4	64	2	8	0.63
				0.5	32	0.63
Gentamicin	P2b-40	4	32	2	8	0.75
				1	16	0.75
Tobramycin	P2b-40	2	32	1	16	1

Although no synergistic interactions were observed, the occurrence of adjuvant interactions in combination therapy is also beneficial and will help reduce the overall dosage required to impart the same level of antimicrobial activity compared to the individual agents. Referring to the definition of therapeutic index, TI, an improvement in TI could be achieved if the MIC is lower and the mammalian cell viability (IC_{50}) are not negatively affected by the potentiation in antimicrobial activity of the combination. To determine if this is the case, the metabolic activity of murine embryonic fibroblast cells (MEFs), which is a measured of cell viability, was determined via alamarBlue assay. Specifically, the metabolic activity of MEFs after 24 h incubation with gentamicin, antimicrobial polymers and combinations thereof was measured and illustrated as gradient square heatmaps in **Figure 4.5.** Evidently, individual homopolymers **P2** and **P2-40** that lack hydrophobic groups are not cytotoxic (ca. 81% and 89% viability,

respectively) even at the highest concentration tested (128 µg mL^{-1}), which is not surprising given that many studies have shown that cationic character alone in polymers is not responsible for inflicting cell death but the presence of hydrophobic groups will by causing physical membrane disruption. Indeed, the random copolymer **P2b-40** which contained 50 mol% of hydrophobic phenyl groups has a low IC$_{50}$ value of 128 µg mL^{-1}. Gentamicin on the other hand was not cytotoxic as expected.

Given the excellent biocompatibility demonstrated by **P2** and **P2-40**, the combinations with gentamicin were equally non-toxic to MEFs. For **P2b-40**, the combinations with gentamicin yield an IC$_{50}$ value of 160 µg mL^{-1}, which was slightly better than that for the polymer alone. This confirms that the biocompatibility of the combination towards mammalian cells is not worse than the individual polymer even though there is a potentiation in antimicrobial activity. The TI values were subsequently calculated through **Equation 1.1** and summarized in **Table 4.3.** The most crucial observation made was the substantial improvement in TI for **P2b-40** in combination with gentamicin compared to **P2b-40** alone, which increased by 4-fold, thus providing confidence that this adjuvant combination is 16 times more likely to target *M. smegmatis* than mammalian cells.
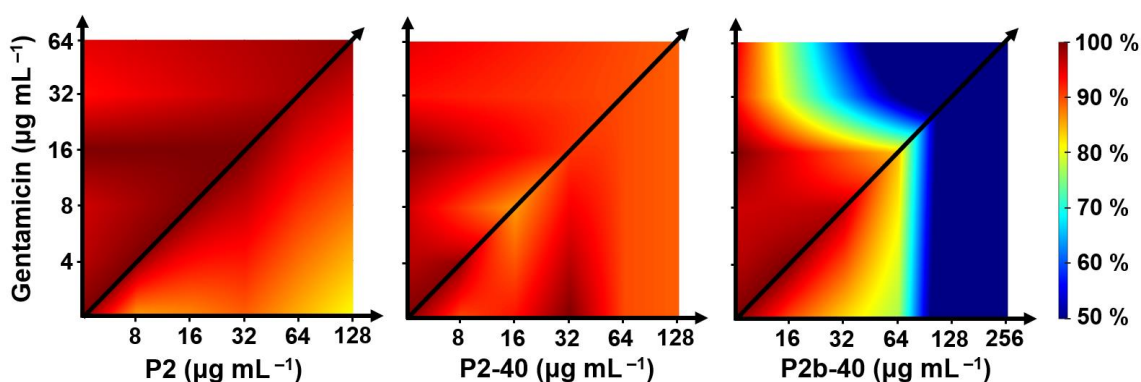
Figure 4.5. The metabolic activity of murine embryonic fibroblast cells after 24 h incubation with

gentamicin (y-axis), antimicrobial polymers (x-axis), and the combinations (diagonal line). The cell

viability, as quantified by the average fluorescent intensity measured at excitation and emission

wavelengths of 550 and 590 nm respectively, is illustrated as a gradient square heatmap generated using

MATLAB. The relationship between the increasing concentration and the change in metabolic activity

was assumed to be linear. The lowest metabolic activity was set to 50% (deep blue) as known as IC50.

The data are based on at least three biological replicates.

Table 4.3. Summary of MIC, IC_{50}, and TI values of antimicrobial polymers and combinations thereof

Antimicrobials	MIC ($\mu g\ mL^{-1}$)	IC_{50} ($\mu g\ mL^{-1}$)	TI
P2	32	> 128	> 4
P2-40	32	> 128	> 4
P2b-40	32	128	4
Gentamicin + P2 (2 + 4)	6	> 192	> 32
Gentamicin + P2-40 (2 + 4)	6	> 192	> 32
Gentamicin + P2b-40 (2 + 8)	10	160	16

4.4 Conclusion

In summary, this chapter described the preliminary investigation of combination therapy

systems involving mycobacteria-targeting antimicrobial polymers and antibiotics. It was

found that both types of quaternary ammonium-containing linear polymers, without (**P2**

and **P2-40**) and with (**P2b-40**) hydrophobic phenyl groups, produced adjuvant

interactions with aminoglycoside antibiotics, namely gentamicin and tobramycin, when

tested against *Mycobacterium smegmatis*. No synergistic interactions were observed

between the polymers and the range of antibiotics screened. In addition, mammalian cell

viability assays performed on murine embryonic fibroblast cells crucially revealed the advantage of combination therapy in improving the therapeutic index of the adjuvant system between **P2b-40** and gentamicin, where the therapeutic value increased from 4 for the polymer alone to 16 for the combination system. The results presented herein could inform future research about novel combination therapy systems (e.g., the synergetic tri-system against mycobacterium) for antimycobacterial applications.

Chapter 5

Conclusions and Outlook

In this thesis, a literature survey (Chapter 2) was presented to highlight key earlier studies pertaining to AMP mimicking polymers and their application in combination therapy with antibiotics against Gram-negative, Gram-positive, and mycobacteria strains. The literature survey highlighted several key advantages of combination therapy, namely the ability to reverse antibiotic resistance in bacteria and the reduced dose required for combination therapy to exert the same antimicrobial activity compared to individual agents. The application of antimicrobial polymers in combination with antibiotics is still relatively new in the field and warrants further investigation. Inspired by this, this thesis explores the development of novel synergetic tri-systems against Gram-negative bacteria (Chapter 3) and adjuvant binary systems against mycobacteria (Chapter 4) using combinations of synthetic cationic antimicrobial polymers and antibiotics.

Despite utilizing different polymers for different bacteria targets, both Chapters 3 and 4 conclusively demonstrated the advantage of combination therapy to not only potentiate antimicrobial activity through synergistic or adjuvant interactions, but also simultaneously improve the therapeutic index (also known as selectivity), thereby shifting the selectivity of the antimicrobial agents towards bacteria targets and minimize toxicity on mammalian cells.

However, the detailed biocompatibility study of the combinational therapy herein was limited due to a lack of in vivo evaluation. The potency of these combinations in vivo requires further evaluation compared to the activity in vitro. Additionally, even recent report shown that the occurrence of synergy is more common for antimicrobial agents

that share the same mechanism,[84] there are still no systematic methods for the discovery of the synergistic effect between polymers and antimicrobial agents.

Research is a continuing endeavour, and as such, several immediate follow up work from this thesis may include investigating the mechanism of action(s) behind the successful combination systems in this thesis through staining and imaging methods to gain fundamental understanding, which may help unearth further newer and more efficient combinations. Additionally, the demonstration of the combination systems in in vivo animal model experiments would instill further confidence on their potential applications in clinical settings. Further, pharmacokinetics and biodistribution studies would also be useful in understanding the efficacy of these combinations in complex multicellular organisms.

All in all, this thesis serves as an excellent guide in informing future research regarding the use of antimicrobial polymers (as well as AMPs) in combination therapy with antibiotics.

Chapter 6

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