Structurally Nanoengineered Peptide Polymers for Combating Multidrug-Resistant Bacteria

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Abstract

Antimicrobial resistance has been named as one of the clinical ‘super-challenges’ of the 21st century. With the rise and prevalence of multi-drug resistant (MDR) ‘superbugs’, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and more recently, the ‘ESKAPE’ pathogens, a return to the pre-antibiotic era is rapidly becoming a reality in many parts of the world. Infections caused by MDR pathogens are a major burden to modern healthcare, as the available treatment options are drastically reduced, leading to increased treatment costs, and high morbidity and mortality rates. However, this growing epidemic of infections caused by MDR pathogens has not been accompanied by an increase in the discovery of novel antimicrobials. In fact, it has been reported that aside from a few narrow spectrum drugs and teixobactin, no new chemical class of antibiotics has appeared in the last 40 years. The challenge remains to develop antimicrobial agents with new mechanisms of action that can overcome acquired resistance without contributing to resistance development.

Over the past few years, our work in drug and gene delivery has demonstrated the potential of star peptide polymers as therapeutic agents, with significant advantages over linear peptides. Building on our prior knowledge, this thesis explores the possibility of using macromolecular engineering techniques to design and develop star peptide polymers that could function as novel antimicrobial agents capable of combating antibiotic-resistant bacteria. Inspired by antimicrobial peptides (AMPs) that form part of the innate immune response in multicellular organisms, 16- and 32-arm star peptide polymers were synthesized, with arms composed of cationic and hydrophobic amino acid moieties co-polymerized in a random fashion. The star polymers were found to demonstrate superior efficacy against clinically-relevant Gram-negative bacteria, including MDR species, compared to their linear ‘one arm’ equivalent and several well-known AMPs, while possessing high therapeutic indices. The lack of any observable bacterial resistance development against the star peptide polymers was attributed to their unique, multi-modal antimicrobial mechanism, which differs from that of antibiotics and AMPs. Based on these attributes, the star peptide polymers were classified as a new class of antimicrobial agents, referred to as ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs).
The subsequent part of this thesis focused on developing further understanding on the structural design and bio-nano interactions of SNAPPs. Through a structure-activity relationship study, the effects of the star arm (co)polymer structure and overall macromolecular architecture on antimicrobial activity and biocompatibility were investigated. Further, the behavior of SNAPPs in physiologically-relevant settings, such as in a bloodstream-mimicking environment, was probed in terms of their antimicrobial efficacy and mode of action. Lastly, this thesis also demonstrated the ability of SNAPPs to synergize with different classes of antibiotics, potentially offering a method to ‘revive’ antibiotics that have already been deemed ineffective.

Collectively, the discovery and development of SNAPPs, as presented in this thesis, represents a breakthrough in the fight against infections caused by antibiotic-resistant bacteria and, hence, a significant advancement in the field of antimicrobial research. This thesis also provides fundamental understanding on the properties and performance of SNAPPs, which will be useful for the optimization of next-generation SNAPPs with enhanced antimicrobial performance and minimal toxic side-effects in vivo. It is thus with hope that this thesis will not only serve as a platform for the development of SNAPPs for actual clinical use, but also act as a stimulus for other researchers in the pursuit of innovative and more effective treatment methods against ‘superbugs’.
Declaration

This is to certify that:

1. This thesis comprises only my original work towards the Ph.D,
2. Due acknowledgement has been made in the text to all other material used,
3. This thesis is less than 100,000 words in length, exclusive of tables, figures, bibliography and appendices.

Shu Jie LAM
November 2016
Preface

1. The text on pages 13-53 is from “Polymeric Nanoparticles for Antimicrobial Applications”, *Progress in Polymer Science, in preparation*.
   - All the work in this publication is my work, completed in collaboration with Edgar H. H. Wong and Cyrille Boyer from the University of New South Wales, Australia.
   - All other co-authors contributed in correcting the work.

2. The text on pages 78-97 is from “Combating Multidrug-Resistant Bacteria with Structurally Nanoengineered Antimicrobial Peptide Polymers”, *Nature Microbiology*, **2016**, *1*, 16162.
   - All the work in this publication is my work.
   - Assistance with polymer synthesis was provided by Sean Lowe.
   - *In vivo* animal models and immune cell phenotyping experiments were conducted with Jason C. Lenzo and Neil M. O’Brien-Simpson.
   - Assistance with gene expression studies was provided by James A. Holden.
   - Cryo-transmission electron microscopy experiments were conducted with Yu-Yen Chen.
   - All other co-authors contributed in correcting the work.

3. The text on pages 98-116 is from “Effects of Polymer Structure on the Antimicrobial Activity and Biocompatibility of Star-Shaped Polypeptides”, *Biomacromolecules, submitted*.
   - All the work in this publication is my work.
   - Assistance with polymer synthesis was provided by Sean Lowe.
   - Assistance with *in vitro* cell experiments was provided by Namfon Pantarat.
   - All other co-authors contributed in correcting the work.

   - All the work in this publication is my work.
• Assistance with membrane permeability assays was provided by Namfon Pantarat.
• Assistance with data analysis was provided by Neil M. O’Brien-Simpson.
• All other co-authors contributed in correcting the work.

• All the work in this publication is my work.
• All other co-authors contributed in correcting the work.
Completing my Ph.D is a significant milestone in my life and this journey is probably one of the most challenging and yet rewarding experiences I have ever encountered. There are many people with whom I have shared my ups and downs with, and to whom I am greatly indebted.

First and foremost, I would like to express my sincere gratitude to my supervisors, Prof. Greg Qiao and A/Prof. Neil O’Brien-Simpson. I would like to thank Greg for recognizing the potential in me to conduct scientific research when I was still an undergraduate student in my penultimate year with no previous research experience. I am grateful to him for providing me with the freedom to pursue and develop my own ideas, yet at the same time, supporting and guiding me throughout the process. To Neil, thank you for your endless support, mentoring and advice throughout my entire research endeavors. Your dedication and enthusiasm to research are exemplary and have inspired me throughout the many years. To both Greg and Neil, I truly appreciate your understanding and support when I was faced with the loss of my father in 2015 and had to take a leave of absence.

Great appreciation is extended to my mentors who have assisted me with my research, Dr. Anton Blencowe, Dr. Adrian Sulistio and Ms. Namfon Pantarat. To Anton and Adrian, thank you for providing me with endless advice, direction, support and encouragement, especially during the initial stages of my Ph.D. Your assistance have greatly contributed to the success of this thesis and the work that resulted in our publication in *Nature Microbiology*. To Namfon (Beth), you sparked my passion for microbiology and your guidance enabled me to complete all the biological assays required for this thesis.

I am also deeply indebted to many scientists and undergraduate research students who have directly or indirectly contributed to the experimental work presented in this thesis. In no particular order, I would like to acknowledge Dr. Jason Lenzo, Dr. James Holden, Dr. Yu-Yen Chen, Mr. Benjamin Hibbs, Dr. Yee-Foong Mok, Dr. Troy Attard, Mr. Sean Lowe, and Mr. Nicholas Jun-An Chan.

I am extremely honored to be a part of the Polymer Science Group and am thankful for the many friendships that have been formed. I would like to thank all past and present members, who have made my Ph.D experience a thoroughly enjoyable and memorable one.
The endless laughers and bonding in the office and laboratory, as well as the annual Christmas gatherings, Kris Kringles, group lunches and the more recent Friday themed dinners will be sorely missed.

I am also very blessed to have received many sources of funding throughout my Ph.D. I would like to acknowledge the Australian government and the University of Melbourne for supporting my studies through the Australian Postgraduate Award (APA) and the International Postgraduate Research Scholarship (IPRS). I am also honored to have received awards and grants in the form of the Eugen Singer Award, the ATA Scientific Young Scientist Encouragement Award, the Melbourne Abroad Travelling Scholarship (MATS), the MRACI Post Graduate Student Travel Bursary, The Messel Travel Bursary, the University of Melbourne Oral Award, the RACI Polymer Student Travel Award for International Polymer Meetings, and the Treloar Prize for Best Oral Presentation - many of which have supported my local and overseas conference travels.

I owe my deepest gratitude to my family and friends, without whom the successful and timely completion of my thesis would not be possible. To my late father, Dr. Lam Pan Nam, to whom I dedicate this thesis to, thank you for being the best dad one could ever wish for. You are the source of my encouragement and inspiration throughout my life, and I look forward to the day that I can finally see you again. To my mum, Mdm. Poh Chou, and my sisters, Xin Jie and Hui Jie, thank you for your prayers and support, and for just being there. To my best friend and partner in life, Edgar Wong, thank you for being my rock and the shoulder that I can lean on. You are always there and ever so willing to walk side by side with me as I go through the ups and downs in life. To all my friends in Australia and Malaysia, especially my ‘Marikhian’ gang and my ex-coursemates, thank you for your continuous support and understanding, even though we are pursuing very different lines of work. The endless gossips and laughter over Whatsapp have comforted me through tough times and made all the stresses of a Ph.D disappear.

Most importantly, all glory and honor be unto the Lord Almighty for His bountiful blessings, love and grace to me and my loved ones.
Publications

Peer-Reviewed Journal Papers (obtained from this thesis)

Chapter 1

“Polymeric Nanoparticles for Antimicrobial Applications” - Invited Review Article

Chapter 2

“Combating Multidrug-Resistant Bacteria with Structurally Nanoengineered Antimicrobial Peptide Polymers”

Chapter 3

“Effects of Polymer Structure on the Antimicrobial Activity and Biocompatibility of Star-Shaped Polypeptides”

Chapter 4

“A Bio-Nano Interaction Study on Antimicrobial Star-Shaped Peptide Polymer Nanoparticles”
Chapter 5

“Star Peptide Polymers Act as a Novel Antibiotic Adjuvant Restoring Antibiotic Sensitivity to Colistin-Multidrug Resistant Bacteria”

Other Publications

“Peptide-Based Star Polymers as Potential siRNA Carriers”

“Biocompatible Single-Chain Polymeric Nanoparticles via Organo-Catalyzed Ring-Opening Polymerization”

“Highly Efficient and Versatile Formation of Biocompatible Star Polymers in Pure Water and Their Stimuli-Responsive Self-Assembly”

“Polypeptide-Based Macroporous Cryogels with Inherent Antimicrobial Properties: The Importance of a Macroporous Structure”

“Star Polymer-Protein Interaction Studies”
“Peptide-Based Star Polymers for Efficient siRNA Delivery”
Shu J. Lam, Adrian Sulistio, Anton Blencowe, Greg G. Qiao
- The 34th Australasian Polymer Symposium (Darwin, Australia), 9 July 2013 (Oral)
- Awarded the Treloar Prize for Best Oral Presentation

“Amphiphilic Star-Shaped Synthetic Polypeptides with Enhanced Antimicrobial Properties”
- The IUPAC World Polymer Congress – MACRO 2014 (Chiang Mai, Thailand), 10 July 2014 (Oral)

“Peptide Stars as High-Potency and Broad-Spectrum Antimicrobial Agents”
- The 6th Australian Society for Medical Research Victorian Student Symposium (Melbourne, Australia), 15 May 2015 (Oral)
- Awarded the University of Melbourne Oral Award

“Antimicrobial Peptide Stars: The Road to Discovery and Development”
- The 250th American Chemical Society National Meeting & Exposition (Boston, Massachusetts, USA), 18 August 2015 (Poster)

“SNAPPing Gram-negative Bacteria with Star-Shaped Polypeptides”
- The 250th American Chemical Society National Meeting & Exposition (Boston, Massachusetts, USA), 19 August 2015 (Oral)
“Combating Multidrug-Resistant Bacteria with Structurally Nanoengineered Antimicrobial Peptide Polymers”
- The 9th Research Symposium for Chinese PhD Students and Scholars in Australia (Melbourne, Australia), 12 November 2016 (Oral)

“Combating Multidrug-Resistant Gram-Negative Bacteria with Structurally Nanoengineered Star-Shaped Peptide Polymers”
- The 36th Australasian Polymer Symposium (Melbourne, Australia), 21 November 2016 (Oral)
# Table of Contents

Abstract ....................................................................................................................................... i  
Declaration ................................................................................................................................ iii  
Preface....................................................................................................................................... iv  
Acknowledgements ................................................................................................................... vi  
Publications ............................................................................................................................. viii  
Conference Proceedings............................................................................................................. x  
Table of Contents ..................................................................................................................... xii  
List of Figures ........................................................................................................................ xvi  
List of Tables ........................................................................................................................ xxiii  
List of Abbreviations .............................................................................................................. xxv  
List of Notations .................................................................................................................... xxx  

## Chapter 1: Introduction ........................................................................................................ 1  
1.1 Background .................................................................................................................... 2  
1.2 Antimicrobial Resistance - Its Causes and Impacts ....................................................... 5  
1.3 Alternative to Antibiotics ............................................................................................... 5  
  1.3.1 Antimicrobial Peptides (AMPs) .............................................................................. 3  
  1.3.2 Synthetic Mimics of AMPs (Linear Polymers) ....................................................... 7  
    1.3.2.1 Peptide-Based Antimicrobial Polymers ........................................................ 8  
  1.3.3 Polymeric Nanoparticles ....................................................................................... 13  
    1.3.3.1 Self-Assembled Polymeric Nanoparticles ..................................................... 14  
      1.3.3.1.1 Polymeric Micelles .................................................................................. 14  
      1.3.3.1.2 Polymeric Vesicles .............................................................................. 28  
    1.3.3.2 Star Polymeric Nanoparticles ................................................................. 33  
    1.3.3.3 Inorganic-Polymer Hybrid Nanoparticles .................................................... 39  
      1.3.3.3.1 Silica-Polymer Core-Shell Nanoparticles .......................................... 39  
      1.3.3.3.2 Metal/Metal Oxide-Polymer Core-Shell Nanoparticles.................... 41  
    1.3.3.4 Other Core-Shell Polymeric Nanoparticles ............................................ 48  
1.4 Current Challenges and Limitations ............................................................................. 53  
  1.4.1 Multidrug-Resistant (MDR) Gram-Negative Bacterial Infections ......................... 54  
  1.4.2 Toxicity .................................................................................................................. 54
Chapter 4: A Bio-Nano Interaction Study on Antimicrobial Star-Shaped Peptide Polymers

4.1 Chapter Perspective
4.2 Introduction
4.3 Results and Discussion
  4.3.1 Antimicrobial Studies in Simulated Body Fluid (SBF)
  4.3.2 Effect of Chelating Agent Addition
  4.3.3 Antimicrobial Studies in Serum
  4.3.4 Effects of Different Biological Environments on Polymer-Bacteria Interactions
4.4 Chapter Summary
4.5 References

Chapter 5: Star Peptide Polymers as an Antibiotic Adjuvant to Restore Antibiotic Sensitivity to Colistin-Multidrug Resistant Bacteria

5.1 Chapter Perspective
5.2 Introduction
5.3 Results and Discussion
  5.3.1 In Vitro Screening for SNAPP-Antibiotic Synergism
  5.3.2 Evaluation of the Degree of Synergy
  5.3.3 Effects of Antibiotic Concentration on Synergism
  5.3.4 Mammalian Cell Toxicity Evaluation
5.4 Chapter Summary
5.5 References

Chapter 6: Conclusions and Future Perspectives

6.1 Conclusions
6.2 Future Perspectives
  6.2.1 Polymer Design and Optimization
  6.2.2 Further Exploration of the Precise Antimicrobial Mechanism
  6.2.3 Translating from Bench to Bedside
6.3 References
Chapter 7: Experimental ................................................................. 161
  7.1 Materials .................................................................................. 161
  7.2 Characterization Methods ............................................................. 162
    7.2.1 Circular Dichroism (CD) ...................................................... 162
    7.2.2 Cryo-Transmission Electron Microscopy (Cryo-TEM) .......... 163
    7.2.3 Dynamic Light Scattering (DLS) ........................................ 163
    7.2.4 Flow Cytometry ............................................................... 163
    7.2.5 Fluorescence Microscopy .................................................. 164
    7.2.6 Gel Permeation Chromatography (GPC) ............................. 164
    7.2.7 Nuclear Magnetic Resonance (NMR) Spectroscopy ............... 164
  7.3 Statistical Analysis ..................................................................... 164
  7.4 General Experimental Methods .................................................. 165
  7.5 Specific Experimental Procedures for Chapter 2 ......................... 170
  7.6 Specific Experimental Procedures for Chapter 3 .......................... 178
  7.7 Specific Experimental Procedures for Chapter 4 ......................... 180
  7.8 Specific Experimental Procedures for Chapter 5 ......................... 182
  7.9 References .............................................................................. 182

Appendices ...................................................................................... 184
  Appendix I Supplementary Information for Chapter 2 ...................... 185
  Appendix II Supplementary Information for Chapter 3 ..................... 234
  Appendix III Supplementary Information for Chapter 5 ................... 243
List of Figures

Chapter 1

Figure 1.1. Mechanisms of antibiotic resistance [2].
Copyright 2013. Reproduced with permission from the Nature Group.

Figure 1.2. a-d. Antimicrobial peptides (AMPs) have been proposed to disrupt the bacterial cell membrane bilayer by different mechanisms [33]. These include the barrel-stave model [35] (a), the carpet mechanism [36] (b), the toroidal pore model [37] (c), and the disordered toroidal pore model [38] (d).
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Figure 1.3. An illustration of the amphiphilic structure of a model antimicrobial peptide (AMP), magainin, and a facially amphiphilic polymer [44]. Hydrophobic and hydrophilic side chains are shown in green and blue, respectively.
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Figure 1.4. The antimicrobial activity of nylon-3 random copolymers was attributed to their ability to adopt globally amphiphilic but irregular conformations when contacted with a bacterial membrane [59].
Copyright 2007. Reproduced with permission from the American Chemical Society.

Figure 1.5. General chemical structure of α-amino acid N-carboxyanhydrides (AANCAs).

Figure 1.6. a-b. Initiation and propagation of the ring-opening polymerization of α-amino acid N-carboxyanhydrides (NCA-ROP) as initiated by primary amines via the normal amine mechanism (NAM) (a) or the activated monomer mechanism (AMM) (b).

Figure 1.7. a. Schematic illustration of a radially amphiphilic antimicrobial peptide (AMP). b, The chemical structure of PHLG-BIm [111].
Copyright 2015. Reproduced with permission from the National Academy of Sciences.

Figure 1.8. The simulated formation of micelles from an amphiphilic peptide obtained from the conjugation of cholesterol, glycine, arginine, and the TAT (YGRKKRRQRRR) peptide [113].
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Figure 1.9. General strategy for the controlled synthesis of cationic amphiphilic polycarbonates via metal-free organocatalyzed ring-opening polymerization (ROP) [85].
Copyright 2011. Reproduced with permission from the Nature Group.
Figure 1.10. Synthetic scheme of random copolymers of MTC-O(CH$_2$)$_3$Cl and MTC-OEt [86].
Copyright 2012. Reproduced with permission from Elsevier Ltd.

Figure 1.11. Illustration of the aqueous self-assembly of cholesteryl cationic polycarbonate oligomers [91].
Copyright 2014. Reproduced with permission from John Wiley & Sons Inc.

Figure 1.12. Illustration of the aqueous self-assembly of PEO-$b$-PCL-$b$-PTBAM copolymer into micelles, which were postulated to interact with bacterial membranes through electrostatic interactions [128].
Copyright 2012. Reproduced with permission from the Royal Society of Chemistry.

Figure 1.13. Synthetic strategy for the PEG-poly(amine acid) copolymers [129].
Copyright 2014. Reproduced with permission from the Royal Society of Chemistry.

Figure 1.14. a. Dissipative particle dynamics (DPD) simulations of the cross-sectional view of PPEG/PQA mixed micelles quaternized with 1-bromobutane (PPEG/PQA4c), 1-bromoocctane (PPEG/PQA8c) and 1-bromododecane (PPEG/PQA12c). b, Schematic illustrations of the mixed micelles [130].
Copyright 2015. Reproduced with permission from the Royal Society of Chemistry.

Figure 1.15. Synthesis of polyion complex (PIC) micelles from TPE sulfonate derivatives (BSTPE or TSTPE) and PEO-$b$-qPDMAEMA block copolymers [132].
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Figure 1.16. The formation of spherical and rod-like assemblies from terephthalamide-polylactide (PLA)-polycarbonate triblock copolymers, and their antimicrobial properties [134].
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Figure 1.17. The formation of antibacterial vesicles from block copolymers of PMEO$_2$MA and PTBAM, and their postulated antibacterial mechanism [156].
Copyright 2013. Reproduced with permission from the Royal Society of Chemistry.

Figure 1.18. Schematic illustration of the self-assembly of polypeptide-grafted chitosan-based nanocapsules, and its ability for drug release upon enzymatic (protease) degradation [159].
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Figure 1.19. Schematic illustration of the structure of TPE$_{core}$P(qDMAEMA-$co$-BMA-$co$-Gd)$_{arm}$ star polymers [186].
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Figure 1.20. Chemical structure of cationic and mannose (man)-functionalized star-shaped polycarbonates [94]. Copyright 2016. Reproduced with permission from John Wiley & Sons Inc.

Figure 1.21. Illustration of the chemical structure of glucosamine-functionalized star polymers [188]. Copyright 2016. Reproduced with permission from the American Chemical Society.

Figure 1.22. Synthetic pathway of qPDMAEMA-decorated silver nanoparticles (AgNPs) [196]. Copyright 2014. Reproduced with permission from the American Chemical Society.

Figure 1.23. Schematic illustration of the synthesis of gold nanoparticles (AuNPs) grafted with PMAG and PMETAI [204]. Copyright 2016. Reproduced with permission from the American Chemical Society.

Figure 1.24. Synthesis of qPDMAEMA-coated Fe$_3$O$_4$ nanoparticles via surface-initiated ATRP (SI-ATRP) [213]. Copyright 2011. Reproduced with permission from the American Chemical Society.

Figure 1.25. Schematic illustration of the synthesis of Fe$_3$O$_4$ nanoparticles coated with an inner PVPS layer and an outer PEDOT shell through layer-by-layer assembly [214]. Copyright 2015. Reproduced with permission from the American Chemical Society.

Figure 1.26. Research theme of the thesis, which is the development of novel antimicrobial agents in the form of star peptide polymers, referred to as ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs).

Figure 1.27. Chapter 2: Chemical structure of antimicrobial star-shaped peptide polymers, referred to as ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs).

Figure 1.28. Chapter 3: Understanding the effects of polymer structure on antimicrobial activity and biocompatibility.

Figure 1.29. Chapter 4: Investigating the bio-nano interactions of star peptide polymers (SNAPPs) in in vivo-mimicking environments.
Chapter 2

Figure 2.1. Synthesis of SNAPPs. Synthesis of SNAPPs via ring-opening polymerization (ROP) of lysine and valine N-carboxyanhydrides (NCAs) was initiated from the terminal amines of poly(amide amine) (PAMAM) dendrimers. Second (G2) and third (G3) generation PAMAM dendrimers (see Supplementary Figure 2.2 for structure of the former) with 16 and 32 peripheral primary amines were used to prepare 16- and 32-arm SNAPPs, respectively. Note that the number of initiating points on the figure does not reflect the actual number which is 16 or 32. The number of repeat units for lysine and valine are $a$ and $b$, respectively. The lysine-to-valine ratios (i.e., $a:b$) are provided in Supplementary Table 2.1.

Figure 2.2. In vivo efficacy of SNAPP S16 in a mouse peritonitis model. a, Schematic of the experimental protocol for the mouse peritonitis model. b–c, Colony forming units (CFU) of A. baumannii (ATCC 19606) (b) and CMDR A. baumannii (FADDI-AB156) (c) found in the peritoneal wash of infected mice 24 h after mock (MEM) treatment or treatment with imipenem (40 mg/kg) or S16 (8.3 mg/kg). d–e, Numbers of peritoneal neutrophils in the mild peritonitis model with mice 24 h after infection with A. baumannii (ATCC 19606) (d) and CMDR A. baumannii (FADDI-AB156) (e) and either mock, imipenem (40 mg/kg) or S16 (8.3 mg/kg) treatments. All data are expressed as mean ± standard deviation as indicated by the error bars, based on values obtained from five biological replicates ($n = 5$). *$P<0.01$, **$P<0.001$, Student’s $t$ test, significant difference from the mock (MEM) control group (b, c) and the imipenem-treated group (d, e).

Figure 2.3. OMX 3D-SIM images of E. coli before and after treatment with AF488-tagged SNAPP S16 in Mueller-Hinton broth (MHB). a–h, Z-projection images of E. coli before (a) and after incubation with AF488-S16 at 0.5 × MBC-tagged (b), 1 × MBC-tagged (c–e), and 2 × MBC-tagged (f–h). Scale bars, 1 µm. The E. coli cell membrane was stained with FM4-64FX (red) and S16 with AF488 (green) in all images. Note that the MBC used refers to the MBC of the fluorescently tagged SNAPP (Appendix I, Supplementary Table 2.11). All images are representative of three independent experiments.

Figure 2.4. Morphological studies of E. coli before and after treatment with S16 in Mueller-Hinton broth (MHB). a–g, Cryo-TEM images of E. coli before (a) and after incubation with S16 for 90 min at a lethal dose of 35 µg/mL (i.e., 1 × MBC of the unlabelled S16 in MHB) (b–g). Large aggregates (possibly aggregates of S16 with medium contents) were observed around the rod-shaped E. coli cells (b–g). Binding of the aggregates to E. coli was observed and the cell membrane appeared disrupted (b). Hole formation (c), OM fragmentation (d), stripping of cell walls and membranes (e), ripping of cell ends (f), and isolated cell fragments (g) were observed. Enlarged images of a–e are provided and the sections enlarged are boxed in red. Scale bars, 200 nm, 250 nm, or 500 nm as indicated. Regions of interest are highlighted by red arrows. All images are representative of three independent experiments.

Figure 2.5. A comparison between the antimicrobial mechanism(s) of typical membrane-disrupting cationic AMPs and the possible mechanism of SNAPPs against Gram-negative bacteria. (a) Cationic AMPs bind to the OM of Gram-negative bacteria via electrostatic interactions, transit across the OM through membrane destabilization, and disrupt the physical integrity of the CM by the ‘barrel-stave’, ‘toroidal-pore’ or ‘carpet' pore

(not shown in figure) mechanisms [12]. (b) SNAPPs, whether in its aggregated or unaggregated state, interact with the OM, PG and CM layers of Gram-negative bacteria via electrostatic attractions and kill the cell by fragmenting/destabilizing its OM and possibly disrupting the CM such that unregulated ion movement is resulted, but also by the induction of the apoptotic-like death pathway (not shown in figure), thereby lysing the cell.

Chapter 3

Figure 3.1. Synthesis of star peptide polymers. Synthesis of the star peptide polymers via ROP of lysine NCA only (SH$_{16}$, SH$_{16}$, SH$_{32}$, and SH$_{32}$) or both lysine NCA and valine NCA (SB$_{16}$, SB$_{16}$, SB$_{32}$, SB$_{32}$, SR$_{16}$, SR$_{16}$, SR$_{32}$, and SR$_{32}$) was initiated from the terminal amines of PAMAM dendrimers. The number of repeat units for lysine and valine are $a$ and $b$, respectively. See also Table 3.1 for the lysine-to-valine ratios (i.e., $a:b$) of copolymers of lysine and valine.

Figure 3.2. CD spectra of SR$_{16}$, SR$_{32}$ and LR (at a concentration of 0.2 mg/mL) in RO water with 80% v/v TFE. The arrows point towards the troughs on the spectra for SR$_{16}$ and SR$_{32}$, which are characteristic of $\alpha$-helices. Arrows with solid line: Characteristic troughs between 205 and 210 nm. Arrows with dotted line: Characteristic troughs at 220 nm. Inset: Expanded view of the CD spectra of LR.

Figure 3.3. Percent hemolysis as a function of peptide polymer concentration. Data are represented as mean ± standard deviation (SD) ($n = 4$).

Figure 3.4. Percent viable HEK293T (a) and H4IE (b) cells as a function of peptide polymer concentration. Viable cells are defined as cells which were PI- and YO-PRO-1-negative. Data are represented as mean ± standard deviation (SD) ($n = 4$).

Chapter 4

Figure 4.1. Synthetic scheme of SNAPPs S16 and S32. Synthesis of SNAPPs via ring-opening polymerization (ROP) of lysine and valine N-carboxyanhydrides (NCAs) was initiated from the terminal amines of poly(amido amine) (PAMAM) dendrimers.

Figure 4.2. 1-N-phenylpahthylamine (NPN) uptake of *E. coli* and *A. baumannii* induced by SNAPP S16. a-b, *E. coli* (a) and *A. baumannii* (b) cells were added to NPN in the presence of increasing concentrations of S16 (0.05-2.92 µM), and NPN fluorescence was monitored starting at $t = 5$ min after the addition of bacterial cells and continued until a maximum is reached (if not at $t = 5$ min). NPN fluorescence was expressed in terms of relative fluorescence units (RFU). Controls whereby no S16 was added (0 µM) were included. The assays were conducted in either SBF (black) or mSBF (red). For *E. coli*, an additional assay was performed in SBF with 1.5 mg/mL of EDTA (blue).
Figure 4.3. Uptake of propidium iodide (PI) by *E. coli* and *A. baumannii* cells before and after treatment with SNAPP S16. a-c, *E. coli* (a, c) or *A. baumannii* (b) cells were incubated with S16 for 90 min at a sub-MIC of 0.09 µM. After incubation the cells were stained with SYTO® 9 and PI nucleic acid dyes prior to analysis. On the two-parameter dot plots obtained by flow cytometry, the x-axis represents fluorescent channel 1 (FL1), which measures the fluorescent emission of SYTO® 9. The y-axis represents fluorescent channel 3 (FL3), which measures the fluorescent emission of PI. Cells were determined to be ‘PI-positive’ if fluorescence emitted is captured by FL3. Controls whereby no S16 was added (0 µM) were included. For *E. coli*, the assays were conducted in MEM (a), SBF (a), mSBF (a), 10% serum in MEM (e), or SBF with 1.5 mg/mL EDTA (c). For *A. baumannii*, assays were conducted in either MEM or SBF (b).

Figure 4.4. Effect of EDTA on antimicrobial activity of SNAPP S16 against *E. coli*. *E. coli* cells were treated with varying concentrations of S16 (0.05-4 µM) and EDTA disodium salt (0.5-2 mg/mL), and the MICs of S16 were evaluated. All data are expressed as mean ± standard deviation as indicated by the error bars (n = 4).

Figure 4.5. Antimicrobial activity of SNAPPs S16 and S32 against *E. coli* in different test media. a-b, MICs were determined for S16 and S32 against *E. coli*. The antimicrobial activity evaluated using 10% SBF (to mimic the ionic composition in 10% serum) or 0.22 mg/mL BSA in MEM (to mimic the albumin concentration in 10% serum) was compared to the MIC in 10% serum (a). The MIC determined in 50% SBF (i.e., same ionic composition as 50% serum) or 1.1 mg/mL BSA in MEM (to represent the albumin concentration in 50% serum) was compared to that obtained when 50% serum was used as the medium (b). The MICs obtained in MEM are included for comparison. All data are expressed as mean ± standard deviation as indicated by the error bars (n = 4).

Figure 4.6. Proposed effects of different medium environments on SNAPP S16-bacterial OM interaction. In MEM, S16 was thought to interact with the LPS layer on the OM via electrostatic interactions and cause OM destabilization and fragmentation (a). Divalent cations, when present in the medium, stabilize the LPS layer and inhibit the ability of SNAPPs to disrupt the OM (b). The addition of a chelating agent restores the membrane disrupting ability of SNAPPs (c). In the presence of serum proteins, the formation of SNAPP-protein aggregates reduces SNAPP-membrane interaction (d).

Chapter 5

Figure 5.1. Chemical structure of SNAPP S16.

Figure 5.2. Screening analysis fjjor synergistic S16-antibiotic pairs. a-f, Log change in colony-forming units (CFU)/mL of *E. coli* (a), *K. pneumoniae* (b), *P. aeruginosa* (c), CMDR *P. aeruginosa* (d), *A. baumannii* (e) and CMDR *A. baumannii* (f) from time zero after treatment for 3 h with S16, a range of antibiotics, and combinations of S16 with the respective antibiotics, with all compounds at their MBC50. All data are expressed as mean ± standard deviation as indicated by the error bars (n = 3). ***P < 0.001, **P < 0.05, Student’s
Figure 5.3. a-e. Log change in CFU/mL of various bacterial species from time zero after treatment for 3 h with S16 at its MBC50 and a range of antibiotics at the indicated concentrations. The synergistic pairs investigated are S16-ampicillin (Amp) against P. aeruginosa (a), S16-AgNO₃ (Ag⁺) against CMDR P. aeruginosa (b), S16-doxycycline (Dox) against CMDR P. aeruginosa (c), S16-AgNO₃ (Ag⁺) against CMDR A. baumannii (d), and S16-imipenem (Imi) against CMDR A. baumannii (e). All data are expressed as mean ± standard deviation as indicated by the error bars (n = 3). ***P < 0.001, **P < 0.05, Student’s t test.

Figure 5.4. Effect of SNAPP S16-Ag⁺ synergistic pair on mammalian cell viability. Percent viability of rat hepatoma (H4IIE) cells after treatment with S16, Ag⁺ (AgNO₃), and co-administration of S16 and Ag⁺ at the indicated concentrations. MBC = 1.6 µM for S16 and 10.6 µM for Ag⁺; MBC_{eff} = 0.8 µM for S16 and 2.9 µM for Ag⁺. Note that the MBC and MBC_{eff} values were taken based on the in vitro activities of the compounds against MDR A. baumannii. All data are expressed as mean ± standard deviation as indicated by the error bars (n = 4).

Appendices

Please refer to Appendices I, II and III for a complete list of supplementary figures.
Chapter 1

Table 1.1. Major antibiotic classes and their mechanisms of action (adapted from [13]).

Chapter 2

Table 2.1. Antimicrobial Activity of SNAPPs and Other Peptides against a Range of Gram-Negative Pathogens.

Chapter 3

Table 3.1. Composition, Molecular Weight and Hydrodynamic Diameter of the Star and Linear Peptide Polymer Library.

Table 3.2. MDCs and MBCs of the Star and Linear Peptide Polymers against \textit{E. coli} and \textit{S. aureus}.

Table 3.3. Biocompatibility of Star and Linear Peptide Polymers (Determined by Hemolysis (HC_{50}) and YO-PRO-1/PI Apoptosis (IC_{50}) Assays).

Chapter 4

Table 4.1. Composition, Molecular Weights and Size of SNAPPs.

Table 4.2. Antimicrobial Activity of SNAPPs against a Range of Gram-Negative Pathogens Tested in Different Media.

Table 4.3. Initial Increase in RFU as a Function of S16 Concentration (Slope of Curve Before Plateau).
Chapter 5

Table 5.1. Evaluation of the Absence/Presence of Synergy between S16 and a Range of Antibiotics Using the Bliss Independence and Highest Single Agent (HSA) Models.

Table 5.2. Fold Reduction of MBC Provided by Synergistic S16-Antibiotic Combinations.

Appendices

Please refer to Appendices I, II and III for a complete list of supplementary tables.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D-SIM</td>
<td>3D-Structured Illumination Microscopy</td>
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<tr>
<td>AANCA</td>
<td>α-Amino acid N-carboxyanhydride</td>
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<tr>
<td>AgNP</td>
<td>Silver nanoparticle</td>
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<tr>
<td>AIE</td>
<td>Aggregation-induced emission</td>
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<td>ALD</td>
<td>Apoptotic-like death</td>
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<td>AM</td>
<td>Acrylamide</td>
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<td>AMM</td>
<td>Activated monomer mechanism</td>
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<td>AMP</td>
<td>Antimicrobial peptide</td>
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<tr>
<td>ATRP</td>
<td>Atom transfer radical polymerization</td>
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<tr>
<td>AuNP</td>
<td>Gold nanoparticle</td>
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<tr>
<td>BIm</td>
<td>1-Methylbenzimidazole</td>
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<tr>
<td>BnCl</td>
<td>Benzyl chloride</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Cbz</td>
<td>Carboxybenzyl</td>
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<tr>
<td>C-PVPS</td>
<td>Catechol-conjugated poly(vinylpyrrolidone) sulfo betaine</td>
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<td>CAC</td>
<td>Critical aggregation concentration</td>
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<td>CD</td>
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<td>CEVE</td>
<td>2-Chloroethyl vinyl ether</td>
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<td>CFU</td>
<td>Colony-forming unit</td>
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<td>CHLG</td>
<td>γ-6-Chlorohexyl-L-glutamate</td>
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<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
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<td>CM</td>
<td>Cytoplasmic membrane</td>
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<td>Critical micelle concentration</td>
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<td>CMDR</td>
<td>Colistin-multidrug-resistant</td>
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<td>CS-g-K</td>
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<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGDMA</td>
<td>Ethylene glycol dimethacrylate</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>G2</td>
<td>Second generation</td>
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<td>Third generation</td>
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<td>GPC</td>
<td>Gel permeation chromatography</td>
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<td>Host defense peptide</td>
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<td>Highest Single Agent</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>Luria broth</td>
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<td>Minimum bactericidal concentration</td>
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<td>Minimum membrane disruptive concentration</td>
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<td>MDR</td>
<td>Multidrug-resistant</td>
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<td>MEM</td>
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<td>MHB</td>
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<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<td>MRSA</td>
<td>Methicillin-resistant <em>S. aureus</em></td>
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<td>mSBF</td>
<td>Modified simulated body fluid</td>
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<td>1-$N$-Phenylnaphthylamine</td>
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<td>Poly[2-(methacyrloyloxy)ethyl trimethylammonium iodide]</td>
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<td>Poly(methyl methacrylate)</td>
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<td>Poly(N-ethylaniline)</td>
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<td>PNIPAm</td>
<td>Poly(N-isopropylacrylamide)</td>
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<td>PPEG</td>
<td>Poly[poly(ethylene glycol) methyl ether methacrylate]</td>
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<td>Poly(phenylalanine)</td>
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<td>Polypyrrole</td>
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<td>PQA</td>
<td>Poly(quaternary ammonium)</td>
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<td>PI</td>
<td>Propidium iodide</td>
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<td>Poly(styrene)</td>
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<td>Poly[3-(triethoxysilyl)propyl methacrylate]</td>
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<td>Poly(trimethylene carbonate)</td>
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<td>Poly(DL-valine)</td>
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<td>PZLL</td>
<td>Poly(Z-L-lysine)</td>
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<td>qPDMAEMA</td>
<td>Quaternized PDMAEMA</td>
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<td>RAFT</td>
<td>Reverse addition-fragmentation chain-transfer</td>
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<td>RBC</td>
<td>Red blood cell</td>
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<td>RFU</td>
<td>Relative fluorescence unit</td>
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<td>Ribonucleic acid</td>
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<td>ROMP</td>
<td>Ring-opening metathesis polymerization</td>
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<td>ROP</td>
<td>Ring-opening polymerization</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SBF</td>
<td>Simulated body fluid</td>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
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<td>SET-LRP</td>
<td>Single electron transfer living radical polymerization</td>
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<td>SI-ATRP</td>
<td>Surface-initiated ATRP</td>
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<td>SLS</td>
<td>Static light scattering</td>
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<td>SNAPP</td>
<td>Structurally nanoengineered antimicrobial peptide polymer</td>
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<td>SPION</td>
<td>Superparamagnetic iron oxide nanoparticle</td>
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<td>YGRKKRRQRRR peptide</td>
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<td>Transmission electron microscopy</td>
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<td>TFE</td>
<td>Trifluoroethanol</td>
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<td>TI</td>
<td>Therapeutic index</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>Trimethylamine</td>
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<td>Trimethyl phosphine</td>
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<td>Ultraviolet</td>
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<td>VBC</td>
<td>Vinylbenzyl chloride</td>
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<td>VDP</td>
<td>Vapor deposition polymerization</td>
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<td>VEH</td>
<td>N-(4-Vinylbenzyl)-N,N-diethylamine hydrochloride</td>
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<tr>
<td>VRE</td>
<td>Vancomycin-resistant <em>Enterococci</em></td>
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### List of Notations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tr>
<td>$D_H$</td>
<td>Hydrodynamic diameter</td>
</tr>
<tr>
<td>$HC_{10}$</td>
<td>Drug concentration required to cause 10% red blood cell lysis</td>
</tr>
<tr>
<td>$HC_{50}$</td>
<td>Drug concentration required to cause 50% red blood cell lysis</td>
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<tr>
<td>$IC_{50}$</td>
<td>Drug concentration required to cause 50% mammalian cell death</td>
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<tr>
<td>MBC50</td>
<td>One-half of the minimum bactericidal concentration</td>
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<tr>
<td>MBC$_{\text{eff}}$</td>
<td>Effective minimum bactericidal concentration</td>
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<td>$M_n$</td>
<td>Number-average molecular weight</td>
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<td>$M_w$</td>
<td>Weight-average molecular weight</td>
</tr>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
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CHAPTER 1

Introduction

1.1 Background

Bacterial infections have long been recognized as the scourge of mankind, and the exploration of newer and more effective treatment and prevention methods remains a continuous endeavor. In the early 20th century, Paul Ehrlich, the winner of the 1908 Nobel Prize in Physiology or Medicine, theorized the concept of a ‘magic bullet’, which is a compound that is able to kill specific microorganisms, such as pathogenic bacteria, with high specificity [1]. In 1928, the world's first antibiotic, penicillin, was discovered by Alexander Fleming as a compound secreted by the fungal species Penicillium rubens [2], and was later developed by Ernest Chain and Howard Florey into a useful therapeutic agent [3]. The landmark discovery by Fleming and co-workers sparked significant interest in the development of antibiotics and initiated a period known as the ‘golden age’ of antibiotic discovery between the 1940s and 1960s [2, 4]. In fact, almost one-half of the antibiotics commonly used today were discovered during that period as natural product extracts (metabolites) isolated from bacteria and fungi [4, 5]. These early antibiotics, such as penicillin and streptomycin, have served humankind well over the last half of the century in fighting deadly infections. However, with the rise and prevalence of antimicrobial resistance, some of these antibiotics – to which the bacteria were originally susceptible to – are no longer effective in today’s world.

By 2050, the recently published Review on Antimicrobial Resistance predicted that drug-resistant infections could cause 10 million deaths worldwide with one death in every three seconds, while costing the global economy up to $100 trillion if no considerable measures are taken [6]. At the moment, resistant infections have resulted in approximately 50,000 deaths annually in the US and across Europe [7]. In Australia, roughly 500 infection cases caused by drug-resistant bacteria are detected each year [8]. Recently, the alarming discovery and emergence of the mcr-1 gene in multiple countries, which harbors primarily in Escherichia coli and renders the bacteria resistant to the ‘last resort’ antibiotic, colistin,
have generated major concerns across the medical circles and added to the urgency of this issue [9].

In this chapter, a succinct background on the rise of antimicrobial resistance will be provided. Subsequently, a comprehensive overview of the alternatives to antibiotic treatment, including the development of antimicrobial peptides (AMPs), synthetic mimics of AMPs and antimicrobial polymeric nanoparticles, will be presented. In addition, this chapter will highlight the current challenges and limitations in antimicrobial therapy, leading to a discussion on the rationale and objectives of the thesis.

1.2 Antimicrobial Resistance – Its Causes and Impacts

Unaware to the general public, antibiotic resistance is an ancient problem and a known natural phenomenon occurring even before modern-day antibiotic clinical use [10, 11]. Bacteria are among the oldest living organisms on Earth. With approximately 2 billion years of existence, they have acquired tools and strategies through evolution to aid in adapting to extreme environments, chemical attacks, surviving with competing microorganisms, and the sophisticated immune defence systems of multicellular organisms. As most antibiotics are extracted from microorganisms such as fungi, algae and bacteria, it is not surprising that pre-existing resistome (i.e., a collection of all antibiotic resistance genes) has been circulating within the microbial pan-genome for hundreds of millions of years [12]. With their short generation times, bacteria are capable of rapidly iterating their genetic material across generations and readily transferring their genes to other bacteria [13]. The fact that most natural product antibiotics are targeted by multiple resistance pathways [14] provides evidence that bacteria have been exposed to these compounds for millennia and have, as a result, evolved highly efficient resistance mechanisms.

While the widespread use of antibiotics in therapeutics and agriculture did not necessarily initiate antimicrobial resistance, it certainly contributed to the prevalence of this issue. When interviewed in 1945 after being awarded a Nobel Prize in Medicine for the discovery of penicillin, Fleming warned that the selection for resistant bacteria could be generated following antibiotic overuse [15]. As a result of the high specificity of antibiotic action (Table 1.1), exposure of bacteria to antibiotics (especially at sub-lethal levels [16]) creates a selection environment for resistant species. Genetic mutations as a counter-measure to antibiotic treatment emerge, and the selected bacterial species with genes for resistance traits proliferates under extended antimicrobial selection while at the same time transferring
its resistance genes to other microbial species [13]. Hence, it is not surprising to note that out of the many antibiotic classes developed over the past 70 years, none has been impervious to antimicrobial resistance [11, 13]. The major types of clinically relevant resistance mechanisms are well-documented (reviewed in [14, 17]) and are summarized in Figure 1.1. These include prevention of drug access to target, target site changes by mutation, non-mutational protection of targets, and direct inactivation or modification of antibiotics. Further, bacteria have also been known to develop tolerance to bactericidal antibiotics by switching on a dormant state (thereby forming a population known as persister cells) [18], or building micro-environmental niches such as biofilms and abscesses [19, 20] – all of which limit the efficacy of existing antibiotics.

**Table 1.1. Major antibiotic classes and their mechanisms of action (adapted from [13])**

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Antibiotic families</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of cell wall synthesis</td>
<td>Penicillins; cephalosporins; carbapenems; daptomycin; monobactams; glycopeptides</td>
</tr>
<tr>
<td>Inhibition of protein synthesis</td>
<td>Tetracyclines; aminoglycosides; oxazolidonones; streptogramins; ketolides; macrolides; lincosamides</td>
</tr>
<tr>
<td>Inhibition of DNA synthesis</td>
<td>Fluoroquinolones</td>
</tr>
<tr>
<td>Competitive inhibition of folic acid synthesis</td>
<td>Sulfonamides; trimethoprim</td>
</tr>
<tr>
<td>Inhibition of RNA synthesis</td>
<td>Rifampin</td>
</tr>
<tr>
<td>Other</td>
<td>Metronidazole</td>
</tr>
</tbody>
</table>
As the majority of antibiotics discovered originate from natural products or their derivatives, antibiotic discovery became stagnated when the screening of available natural resources was exhausted in the 1960s [21]. Since then, the discovery of new antibiotics remained few and far between, and most new antibiotics discovered after the 1970s were merely variants of existing antibiotic families [11]. These new derivatives were only effective temporarily and eventually became susceptible to antimicrobial resistance [22]. Since resistance acquisition is inevitable and antibiotics gradually lose their effectiveness over time, pharmaceutical companies became disinterested in developing new antibiotics primarily due to the unfavourable economics compared to investments in other fields, such as cancer therapy and anti-inflammatory medications. As of September 2015, an estimated 39 new antibiotics are in clinical development with only two candidates belonging to new antibiotic classes [7].

In order to mitigate the limitations of natural product antibiotics, various research groups have been focusing on the development of fully synthetic antibiotic molecules with the hope that these compounds should offer chemical scaffolds that are less likely to succumb to pre-existing resistance. However, this approach was not particularly successful as the
chemical scaffolds used were often selected from existing drug libraries (such as those for cancer treatment) and might not be targeted to bacteria [11]. Recently, Myers and co-workers reported the development of a fully synthetic platform for the preparation of macrolide antibiotics, which utilizes the convergent assembly of simple chemical building blocks to enable the synthesis of diverse and novel macrolide structures not previously attainable through traditional synthetic methods [23]. While some of the compounds demonstrated efficacy against clinical bacterial strains that are resistant to known macrolides, further investigations are still needed to determine if resistance will eventually be acquired against these macrolide derivatives.

1.3 Alternatives to Antibiotics

In parallel to the on-going development of antibiotics based on ‘traditional’ antibacterial targets through synthetic methods, a relatively new research endeavour towards antimicrobial approaches based on new compound classes and non-traditional targets has emerged in recent years. Most research in this area aims to explore antimicrobial mechanisms that are fundamentally different to that of antibiotics. Based on the understanding that resistance development against antibiotics is inevitable due to the target-specific nature of antibiotics, extensive research has been conducted on a unique class of peptides known as antimicrobial peptides (AMPs), which target bacterial cell membranes and hence possess broad-spectrum activity. Taking inspiration from the structure and function of AMPs and with the advent of controlled polymerization techniques, polymers with inherent antimicrobial properties have been developed in the form of linear polymers as well as nanoparticles. It is noteworthy that other approaches such as quorum-sensing targeting [24] and phage therapy [25] are also being pursued, but are mostly still at their infancy compared to AMPs and AMP-inspired polymers.

1.3.1 Antimicrobial Peptides (AMPs)

AMPs are naturally evolved host defense peptides (HDPs) and form part of the innate immune response of most multicellular organisms [26]. AMPs have been shown to demonstrate broad and effective antimicrobial activity against bacteria, viruses and fungi [27]. While a plethora of naturally-occurring AMPs exists, they typically comprise 10 to 50 amino acids with cationic and hydrophobic moieties [26]. Unlike antibiotics, it is commonly understood that the lethality of most AMPs relies on physically damaging the bacterial cell membrane [28]. Even for AMPs that were found to interact with intracellular targets, initial
peptide-membrane interactions were found to be the determinant for activity [29]. The membrane-disrupting ability of AMPs is often attributed to their amphiphilic structure, which promotes selectivity for microbial membranes and facilitates interactions with the membrane bilayer [30]. Due to the cationic nature of AMPs, ideally, they would preferentially bind to bacterial surfaces, which have a higher net negative charge, compared to mammalian cell membranes, which are more zwitterionic [26, 31]. This is followed by diffusion through the bacterial membranes, causing membrane disintegration (of which the different mechanisms are reviewed in [32, 33]; see Figure 1.2 for a summary), leakage of cellular components, and lastly cell death [34].

Figure 1.2. a-d. Antimicrobial peptides (AMPs) have been proposed to disrupt the bacterial cell membrane bilayer by different mechanisms [33]. These include the barrel-stave model [35] (a), the carpet mechanism [36] (b), the toroidal pore model [37] (c), and the disordered toroidal pore model [38] (d). Copyright 2009. Reproduced with permission from the Nature Group.

While the mode of action of AMPs endows them with broad-spectrum activity and a lower tendency for resistance acquisition (although AMP-resistant species have been reported [39]), AMPs still face several practical challenges en route to clinical usage. These include (1) high cytotoxicity due to their low selectivity for bacteria over mammalian cells, (2) susceptibility to proteolytic attack leading to short half-lives in vivo, (3) reduction in efficacy under physiological conditions, and (4) poorly understood pharmacokinetics [27, 40].
Consequently, most of the AMPs considered for systemic administration failed at pre-clinical stage and a majority of the pharmaceutical efforts so far have been focused on the development of AMPs for topical applications [41]. Despite these drawbacks, the preparation of synthetic AMPs (as mimics of naturally-occurring AMPs) using solid phase peptide synthesis (SPPS) remains an active research area, with increasing emphasis placed on the synthesis of non-naturally occurring analogues (composed of D-α- amino acids, β-peptides, or peptoids) in order to engineer AMPs with enhanced properties [42]. However, the reliance on SPPS, which is typically laborious and low-yielding, for the synthesis and optimization of AMPs remains a major hurdle and complicates their effective usage [42].

1.3.2 Synthetic Mimics of AMPs (Linear Polymers)

The concept of antimicrobial synthetic polymers was first inspired by the discovery of naturally occurring polymers such as chitosan and poly-ɛ-lysine that are inherently antimicrobial [43]. Synthetic polymers currently being studied for antimicrobial applications are mostly polycations containing quaternary ammonium, phosphonium, sulfonium, or guanidine groups to exploit the electrostatic interactions with the anionic bacterial cell wall [27]. In fact, in an attempt to mimic the structure of AMPs, most antimicrobial polymers are designed as cationic and amphiphilic systems in the form of block or random copolymers from a combination of hydrophilic and hydrophobic monomers [31].

Rigorous research on antimicrobial polymers was initiated when Tew and co-workers pioneered the synthesis and development of inexpensive polymers and oligomers that mimic the amphipathic structure of α-helical AMPs such as magainin, which is thought to be important for their membrane-disrupting ability [44]. Polymers were designed to adopt facially amphiphilic conformations, in which the cationic/hydrophilic and hydrophobic side chains segregate onto opposing regions or faces (Figure 1.3). The polymers were synthesized from facially amphiphilic monomers and included polyacrylamides [44, 45], poly(phenylene ethynylene)s [46-50] and polynorbornenes [51-58], where pronounced antimicrobial activities and high bacteria selectivities were demonstrated in most cases. It was later proposed and demonstrated by Gellman and co-workers that sequence-random polymers with cationic and hydrophobic subunits are capable of adopting membrane-induced irregular conformations that are globally amphiphilic in nature (Figure 1.4) [59]. Based on this theory, amphiphilic nylon-3 random copolymers through the ring-opening polymerization (ROP) of β-lactams
were synthesized as mimics of AMPs and were shown to have good antimicrobial efficacies [59-63].

![Figure 1.3](image1.png)

**Figure 1.3.** An illustration of the amphiphilic structure of a model antimicrobial peptide (AMP), magainin, and a facially amphiphilic polymer [44]. Hydrophobic and hydrophilic side chains are shown in green and blue, respectively. Copyright 2002. Reproduced with permission from the National Academy of Sciences.

![Figure 1.4](image2.png)

**Figure 1.4.** The antimicrobial activity of nylon-3 random copolymers was attributed to their ability to adopt globally amphiphilic but irregular conformations when contacted with a bacterial membrane [59]. Copyright 2007. Reproduced with permission form the American Chemical Society.

Over the years, a wide range of synthetic antimicrobial polymers was developed, such as poly(vinyl pyridine)s [64], poly(vinyl alcohol)s [65], poly(vinyl ether)s [66], polystyrenes [67], polyacrylates [26, 34, 68-83], polymethacrylamides [84], and polycarbonates [85-95] – most of which are based on the copolymerization of a cationic (hydrophilic) monomer and a hydrophobic co-monomer. Despite their promising antimicrobial activities, these polymers are mostly non-biodegradable (with the exception of polycarbonates) and in some cases, exhibit toxicity to human or mammalian cells, which render them unsuitable for *in vivo* applications. Hence, significant interest has been generated towards the development of peptide-based antimicrobial polymers, which will be thoroughly reviewed in the following section.

**1.3.2.1 Peptide-Based Antimicrobial Polymers**

The incorporation of amino acid building blocks into synthetic precursors to create hybrid polymer systems has been an increasingly popular strategy used to impart
biodegradability and biocompatibility to the polymer construct [96]. Aside from the fact that amino acids are naturally present in the human body, the plethora of side-chain functional groups present on these (bio)molecules opens up a convenient route for the introduction of functionalities such as acid, amine, thiol, and indole [96], thereby facilitating the synthesis of diverse and complex polymer structures with tunable properties. Additionally, the synthesis of amino acid-based polymers is of particular interest as this could potentially lead to novel polymeric systems which possess the advantageous properties of amino acids, while avoiding the limitations of SPPS through the use of controlled polymerization techniques.

Following Leuchs’ discovery of α-amino acid N-carboxyanhydrides (AANCAs) (Figure 1.5) in 1906 [97] and the first account of well-controlled and living polymerization of AANCAs by Deming in 1997 [98], the ROP of AANCAs (hereby abbreviated as NCA-ROP) has been viewed as a viable controlled polymerization method for the synthesis of well-defined polypeptides. This technique started to gain traction over the traditional approach of peptide synthesis, SPPS, as NCA-ROP involves simple reagents and is able to prepare high molecular weight polymers in good yields and large quantities without racemization at the chiral centres [99]. NCA-ROP can be initiated by nucleophiles like primary amines and proceed via the normal amine mechanism (NAM) or the activated monomer mechanism (AMM), as shown in Figure 1.6. Over the years, extensive work has been done to devise optimized pathways which would lead to more controlled polymerization producing more well-defined, high molecular weight polypeptides with higher end-group fidelity. These included studies on the use of transition metal- [98, 100], hydrochloride salt- [101], or silazane-based initiators [102], and adjustments to the reaction conditions, such as by using high vacuum techniques [103] or low reaction temperatures [104]. Collectively, the remarkable progress made in controlled NCA-ROP opened doors to the exploration of the use of functional synthetic polypeptides in numerous medical and pharmaceutical applications, notably in the areas of drug and gene delivery [105, 106].

![General chemical structure of α-amino acid N-carboxyanhydrides (AANCAs).](image)

Figure 1.5. General chemical structure of α-amino acid N-carboxyanhydrides (AANCAs).
In the field of antimicrobial research, NCA-ROP offers great potential as an efficient and cost-effective method for the large-scale synthesis of AMPs. Despite the lack of precise sequence control as compared to SPPS, the antimicrobial properties of linear, AMP-mimicking polypeptides synthesized through NCA-ROP were suggested in 2001. In a structure-activity relationship study conducted by Wyrsta et al., block and random copolypeptides composed of lysine copolymerized with a hydrophobic amino acid monomer (leucine, phenylalanine, isoleucine, valine, or alanine) were screened for membrane activity using lipid vesicles of different compositions to mimic bacterial and mammalian cell membranes [107]. Polymers with high hydrophobicity and intermediate chain lengths, similar to AMPs, were found to be membrane-active in general, with copolymers containing lysine and leucine demonstrating the highest membrane activity. The extent of membrane interaction was also found to be dependent on lipid composition (charge) as well as peptide secondary structure. While the investigation was not conducted using biological cells, it demonstrated the ability of NCA-ROP to afford peptides with precisely controlled chain length and composition which could potentially yield potent yet non-hemolytic mimics of naturally-occurring AMPs.
In 2010, Chan-Park and co-workers successfully demonstrated the first synthesis of AMPs via NCA-ROP [108] using a nickel-based initiator. Similar to the earlier work by Wyrsta et al. [107], lysine was selected as the cationic component while the hydrophobic co-monomer was chosen from alanine, phenylalanine, or leucine. A range of polypeptides was synthesized with varying hydrophobic contents and lengths, and the polymers were subsequently tested against Gram-negative bacteria (i.e., Escherichia coli, Pseudomonas aeruginosa, and Serratia marcescens), Gram-positive bacteria (Staphylococcus aureus), and fungi (Candida albicans) to determine their minimum inhibitory concentrations (MICs, which are the minimum drug concentrations required to inhibit microbial growth). Despite the absence of any distinct secondary structure, two variants (i.e., poly(lysine10-co-phenylalanine7.5-leucine7.5) and poly(lysine10-co-phenylalanine15)) exhibited the lowest MICs (31-250 µg/mL), even surpassing the efficacy of selected AMPs such as LL-37, indolicidin, and magainin I. However, the membrane activity of these synthetic AMPs was not bacterio-selective, as the two best performing copeptides were also highly hemolytic within the therapeutic concentration range. This prohibited the development of these antimicrobial polypeptides as therapeutic agents.

In order to prepare AMPs with high hemo- and cytocompatibility, Engler et al. utilized the ROP of γ-propargyl-L-glutamate NCAs to form a clickable polypeptide backbone with varied lengths of 30 to 140 [109]. Different side group functionalities were introduced through the copper-catalyzed alkyne-azide cycloaddition click reaction to produce polypeptides with primary, secondary, tertiary, or quaternary amines with various hydrophobic pendant groups. The study was aimed at investigating the effects of different side chains (amines and hydrophobic groups) on antibacterial activity against E. coli and S. aureus, where the polypeptides were either tested as free polymers in solution or coated on glass substrates. Generally, the polymers were found to display moderate antimicrobial efficacies which increased with increasing hydrophobicity, as well as low hemolytic activities.

In an attempt to boost the biocompatibility of synthetic peptide-based antimicrobial polymers without compromising their bactericidal activity, a class of polymers – termed cationic peptidopolysaccharides – was developed by Li et al. [110] These polymers were designed to mimic the structure of the peptidoglycan (PG) layer found in the cell wall of all bacteria by forming polypeptide-grafted chitosan copolymers. Interestingly, increased efficacy was observed when chitosan was grafted with homopolymers of lysine (CS-g-K), instead of the AMP-mimicking lysine-phenylalanine copolymers. The authors postulated that
the cationic charge of poly(lysine) and the hydrophobicity of the chitosan backbone were sufficient to promote antibacterial activity. The polymer CS-g-K16 exhibited excellent activity against *E. coli*, *P. aeruginosa*, *S. aureus*, *C. albicans*, and *Fusarium solani*, with MICs ranging from 5 to 20 µg/mL where activity was attributed to membrane disruption and pore formation. Furthermore, as all CS-g-K variants were relatively non-hemolytic, a high selectivity index (HC50/MIC, where HC50 is the drug concentration required to cause 50% red blood cell lysis) of greater than 10,000 was observed for CS-g-K16. It was further demonstrated that the polymer exhibited negligible toxicity towards mammalian cells both *in vitro* and *in vivo*.

More recently, Cheng and co-workers proposed an innovative approach to synthesize antimicrobial polypeptides with enhanced properties [111]. Cationic, helical homopeptides with a radially amphiphilic structure (*i.e.*, a hydrophobic, helical core and a charged, hydrophilic shell – see Figure 1.7a) were prepared from the ROP of γ-6-chlorohexyl-L-glutamate (CHLG) NCA monomers, followed by amination with 1-methylbenzimidazole (BIm) to form PHLG-BIm (Figure 1.7b). It was envisaged that the radially amphiphilic structure, where the hydrophobic core is shielded by the cationic shell, would promote binding to bacteria, reduce non-specific interactions and self-aggregation, and lead to better proteolytic stability. As hypothesized by the authors, the radially amphiphilic and helical polymer, PHLG-BIm40, showed excellent efficacy (MIC 1.5-26.1 µM) against a range of Gram-positive and Gram-negative species, including methicillin-resistant *S. aureus* (MRSA) and clinically isolated *Helicobacter pylori* strains. Activity was retained even in the presence of different simulated physiological conditions, such as in the presence of salts, serum, plasma, mucin, or after extended incubation with proteases. Further, PHLG-BIm40 was also shown to exhibit high selectivity (>32) against *E. coli* DH5α as compared to its non-helical analogue (>2), demonstrating the importance of radial amphiphilicity in boosting the performance of AMPs.
1.3.3 Polymeric Nanoparticles

The possible enhancement of antimicrobial activity through nanoparticle formation was inspired by multivalent interactions found ubiquitously throughout biology, where simultaneous binding of multiple ligands on one entity to multiple receptors on another could lead to much stronger effects than corresponding monovalent systems [112]. The hypothesis was substantiated by studies reported by Yang and co-workers in 2009 and 2010, where core-shell micellar nanoparticles based on the self-assembly of an amphiphilic peptide (prepared by SPPS) showed superior efficacy against a range of Gram-positive bacterial and fungal species (Figure 1.8) [113, 114]. Improved antimicrobial properties in the assembled state were attributed to increased and more localized density of cationic charges and peptide mass, resulting in stronger electrostatic interactions with anionic microbial membranes.

In this section, synthetic polymer-based nanoparticles that displayed direct antimicrobial activity will be reviewed. These polymeric nanoparticles are categorized based on their complex macromolecular architecture, and the effect(s) of polymer architecture on the antimicrobial performance and biocompatibility of such nanoparticles will be discussed.
Self-assembly has been recognized as one of the most commonly used route for the construction of nanostructured materials from small building blocks [115]. Inspired by nature, the formation of highly complex molecular and supramolecular structures in the most thermodynamically stable form, such as micelles and vesicles, is made possible by multiple weak and non-covalent interactions between the chemical building blocks [115-117]. In the field of therapeutics, self-assembly has been increasingly used for the synthesis of nano-sized biomaterials due to the relative ease, precision and versatility of the method, enabling the incorporation of functions such as stimuli-responsiveness, recognition and targeting [118]. More specifically, micelles and vesicles formed through the self-assembly of polymer building blocks have been explored for possible use as novel antimicrobial agents.

1.3.3.1 Polymeric Micelles

Polymeric micelles, which are core-shell nanoparticles typically formed through the self-assembly of amphiphilic block copolymers [119], are regarded as one of the most extensively studied nanostructure type for antimicrobial applications. This is possibly contributed by the fact that such nanostructures have been the focus of numerous studies in drug delivery for the past three decades [119]. Interestingly, with respect to polymeric micelles that are inherently antimicrobial, the role of micellization on antimicrobial activity has been frequently debated. The following discussion will be divided into two categories, where studies that dismissed the
positive contributory role of micellization on antimicrobial activity will be firstly presented [66, 88, 120-126], followed by reports that suggested the importance of self-assembly in promoting efficacy [85, 86, 91, 127-135].

Waschinki et al. prepared a series of poly(2-alkyl-1,3-oxazoline)s through living cationic polymerization, where the types of polymer backbone, starting end-groups (also known as satellite groups) and terminal biocidal functional groups were varied [120]. As polyoxazolines have been known to be low-fouling and bacteria-repelling [136, 137], bactericidal properties were imparted through the introduction of bactericidal end-groups. The MICs of the functionalized polyoxazolines ranged from 0.2 to 4.7 mg/mL (equivalent to 0.08-0.94 mM) against *S. aureus*. While the polymers were capable of self-assembly, the authors did not observe any distinct trend between the critical micelle concentrations (CMCs) of the polymers and their MICs. Instead, the experimental results suggested that the chemical structure of the polymers, including the nature of the backbone and the type of satellite groups, seemed to be of greater influence towards antimicrobial activity. In a follow-up study by the same research group, the role played by the satellite groups in determining the antimicrobial performance of poly(2-alkyl-1,3-oxazoline)s against *E. coli* and *S. aureus* was validated, where polymer self-assembly into micelles was again demonstrated to be redundant for antibacterial activity [121]. In fact, the results suggested that micellization was detrimental to biological activity against both Gram-positive and Gram-negative bacteria, as polymers with low CMCs were less antibacterial than those with higher CMCs. The authors postulated that the polymers might be more active as free linear chains in solution and hence the formation of supramolecular structures could reduce the polymer chain mobility. Through static light scattering (SLS), nuclear magnetic resonance (NMR) analysis and membrane interaction studies, the authors concluded that aggregation processes are not a major determinant for antibacterial efficacy for the polyoxazoline system investigated [122].

A similar observation was made by Kiss et al., where a study was conducted to investigate the structure-activity relationship of cationic polyelectrolytes, in the form of branched poly(ethylene imine)s (PEIs) functionalized with various quaternary ammonium compounds and alkyl groups, with bacteria [123]. Through *in vitro* antibacterial susceptibility tests and model membrane experiments, the authors observed a trend between antibacterial behaviour and polymer hydrophobicity, where the activity and membrane binding ability of the modified PEIs decreased with increasing hydrophobicity. This observation was attributed to a stronger tendency for micellization for the PEIs that were more hydrophobic, where the
hydrophobic components required for microbial membrane interactions were shielded within the core, therefore hampering the binding between the polymer and membrane lipid layers.

In other polymeric systems such as polyvinyl ethers [66], polymethacrylates [124], polycarbonates [88], and polyacrylates [125, 126] where the polymers synthesized were able to self-assemble into micelles, the notion of self-assembly as a prerequisite for antimicrobial activity was also dismissed. Kuroda and co-workers developed a library of amphiphilic block and random vinyl ether copolymers using base-assisting living cationic polymerization, where all copolymers synthesized displayed bactericidal activity against E. coli and hemolytic behaviours at much lower concentrations than their critical aggregation concentrations (CACs) [66]. The authors observed that the copolymer structure and conformation as individual polymer chains were more crucial than macromolecular assembly in determining biological activity. By performing molecular dynamics simulation to investigate the interactions of multiple antibacterial, random and amphiphilic methacrylate-based copolymers with model bacterial membranes, the same research group postulated that while the formation of micelle-like structures in solution phase could promote faster binding with bacterial membranes, the polymer aggregates would dissociate into single polymer chains upon contact with bacterial membranes due to more favorable polymer-membrane lipid interactions compared to weaker intermolecular bonds within the micelles [124].

In a polycarbonate-based system reported by Engler et al., a library of homopolymers and statistical copolymers with pendant quaternary ammonium groups separated from the backbone by a hydrophobic spacer was developed [88]. While the self-assembling abilities of all polymers were demonstrated, their MICs against a range of Gram-positive and Gram-negative bacteria were lower than their CMCs, suggesting that bacterial growth inhibition was effected by the polymers as individual chains. Additionally, the authors concluded that the balancing of charge and hydrophobicity was more important in optimizing antibacterial activity.

With the ultimate goal of fabricating dual-functionalized polymers with antibacterial and antioxidative properties to combat bacterial biofilms, micelles through the self-assembly of cationic and amphiphilic random polyacrylates bearing tertiary amine (N,N-diethylethlenediamine) and hydrophobic catecholic (hydroxytyrosol) side chains were prepared [125, 126]. Besides being a well-known antioxidant, the activity of hydroxytyrosol against certain Gram-positive species has been reported [138]. The combination of both
tertiary amine and hydroxytyrosol moieties at an optimal ratio resulted in antibacterials that were effective against *Staphylococcus epidermis* and possessed low hemolytic activities [125]. However, a comparison of their MICs and CMCs revealed that the copolymers acted against bacteria as single chains and not micelles [126]. It was further elucidated by molecular dynamics simulation that the antibacterial copolymers displayed single chain folding behaviours, which were postulated to promote exposure of the cationic groups and facilitate electrostatic interactions with the anionic bacterial cell membranes.

To the best of our knowledge, the contributory role of polymeric micelle formation on antimicrobial activity was first suggested by Lenoir *et al.* through self-assembled micelles formed by poly(ethylene-co-butylene)-b-poly[2-(dimethylamino)ethylmethacrylate] (PEB-b-PDMAEMA) block copolymers that were quaternized with octyl bromide [127]. The polymers synthesized were found to have a bimodal size distribution with two populations bearing hydrodynamic diameters ($D_H$) of 25 and 85 nm present, where the larger of the two populations was speculated to be micellar aggregates. Efficient activity against *E. coli* that resulted in a 4-log (99.99%) reduction in cell counts within 30 min at a polymer concentration of 100 µg/mL was demonstrated. The antibacterial properties of the block copolymers were attributed to micellization, although the relationship between polymer CMCs and effective antibacterial concentrations was not clearly shown.

In 2011, a notable study was published by Yang and co-workers, where the first biodegradable antimicrobial polymeric nanoparticles in the form of micelles were reported (Figure 1.9) [85]. The micelles were self-assembled from amphiphilic triblock polycarbonates synthesized *via* metal-free organocatalyzed ROP, which resulted in a central poly(5-methyl-5-(3-chloropropyl) oxycarbonyl-1,3-dioxan-2-one) (PMTC-O(CH$_2$)$_3$Cl) block sandwiched between two poly(trimethylene carbonate) (PTMC) blocks. The PMTC block was subsequently quaternized with trimethylamine to render it cationic for electrostatic interactions with bacterial cell membranes. Depending on the length of the PMTC and PTMC blocks, micelle-like nanoparticles ranging from 43 to 402 nm in diameter were fabricated. The nanoparticles exhibited MICs between 4.3 and 10.8 µM against a range of Gram-positive bacteria, including *Bacillus subtilis*, *Enterococcus faecalis*, *S. aureus*, and MRSA, and *Cryptococcus neoformans*, a fungal species. It is noteworthy that the MICs of the best-performing nanoparticles were all above their CMCs, which indicated an obvious link between micellization and antimicrobial efficacy. The nanoparticles were confirmed to be microbicidal as their minimum bactericidal concentrations (MBCs) corresponded to the MICs.
By imaging the bacterial cell morphology post-treatment using transmission electron microscopy (TEM), the nanoparticles were shown to effect their antimicrobial action by cell wall and membrane disruption as well as cell lysis. In addition to the low hemolytic behaviours of the polycarbonate micelles, the authors demonstrated that the nanoparticles did not induce significant toxicity to mice. Despite the significant achievements of this study, the inability of the nanoparticles to combat Gram-negative bacteria was recognized as a drawback. Gram-negative bacteria has been recently viewed as a more critical healthcare issue than Gram-positive bacteria, primarily due to a shortage in effective and biocompatible drugs targeting resistant Gram-negative bacteria [139, 140].

In order to endow polycarbonate-based micelles with the capability to kill both Gram-positive and Gram-negative bacteria, Yang and co-workers devised a series of micelles based on random copolymers of MTC-O(CH$_2$)$_3$Cl and 5-methyl-5-ethyloxycarbonyl-1,3-dioxan-2-one (MTC-OEt) monomers (Figure 1.10) [86]. Similar to their previous study [85], quaternization using trimethylamine was performed on the resulting copolymers. The authors hypothesized that a random copolymer design would result in micelles with more accessible hydrophobic moieties compared to a block copolymer structure, such as that used in their previous study [85] where the hydrophobic parts are shielded in the core, therefore increasing the likelihood of membrane insertion and disruption induced by the hydrophobic components. This hypothesis was later confirmed through imaging experiments, from which the random
copolymers were validated to be more efficient at membrane disruption than block copolymers. Growth inhibitory effects (MICs within 63-250 µg/mL) were displayed by some of the polycarbonates against both Gram-positive (S. aureus) and Gram-negative species (E. coli and P. aeruginosa), and self-assembly was again demonstrated as necessary for activity. Interestingly, by varying the molecular weights of the copolymers, it was shown that a lower overall molecular weight facilitated better activity against S. aureus, while higher molecular weight polymers were more effective against the Gram-negative species. This distinction was attributed to the morphological differences of Gram-positive and Gram-negative bacterial cell membranes, where the former possesses a thick outermost peptidoglycan layer known to entrap higher molecular weight polymers [141]. In terms of nanoparticle biocompatibility, the polymers displayed minimal hemolytic effects over a wide range of concentrations, where the selectivity (determined using the ratio of polymer concentration that caused 50% red blood cell lysis, HC₅₀, to MIC, i.e. HC₅₀/MIC) of the polymers with the highest antibacterial activities was greater than 16 towards both Gram-negative and Gram-positive species.

Figure 1.10. Synthetic scheme of random copolymers of MTC-O(CH₂)₃Cl and MTC-OEt [86]. Copyright 2012. Reproduced with permission from Elsevier Ltd.

As their previous study indicated the importance of polymer-membrane integration in enhancing antimicrobial activity [86], Yang, Hedrick and co-workers investigated the effect of incorporating cholesterol in polycarbonate oligomers [91]. Besides its well-known membrane assimilation characteristic, cholesterol also possesses the propensity for rotative face-on-face stacking [142] which would promote self-assembly when conjugated to a polymer or oligomer. Using different cholesteryl initiators, a cyclic carbonate monomer, MTC-BnCl, was polymerized to varying degrees of polymerization (DPs) and further
subjected to quaternization by either trimethylamine (TMA) or trimethyl phosphine (TMP). The resulting polymers were found to self-assemble in water into 10-11 nm coin-shaped micelles (Figure 1.11). Compared to analogous polycarbonates without any cholesteryl groups, the cholesterol-functionalized polymers displayed enhanced activities against *S. aureus*, *E. coli* and *P. aeruginosa*, while the hemolytic effects remained similar as before. Oligomers with DPs above 10 generally had lower MICs which resulted in higher selectivities (HC$_{50}$/MIC). Depending on the hydrophobic-hydrophilic balance, MICs ranging from 3.9 to 250 µg/mL were attained by the optimized oligomers against *S. epidermis*, *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans* (i.e., a fungal species). Noteworthy, a model oligomer with a selectivity of 13 against *E. coli* did not induce any observable resistance in *E. coli* even after 19 passages, and was further shown to be effective against drug-resistant *E. coli*. However, it should be noted that several oligomers in this study were found to be antimicrobial as individual chains (rather than as self-assembled structures), as they registered MICs that were less than their CMCs.

![Figure 1.11. Illustration of the aqueous self-assembly of cholesteryl cationic polycarbonate oligomers [91]. Copyright 2014. Reproduced with permission from John Wiley & Sons Inc.](image)

Following the earlier reports on polycarbonate-based micelles by Yang and co-workers [85, 86], increasing interest has been generated in the development of antimicrobial, self-assembled micelles using other polymer systems. For example, antibacterial and
biodegradable poly(ethylene oxide)-b-poly(ɛ-caprolactone)-b-poly[(2-tert-butylaminoethyl) methacrylate] (PEO-b-PCL-b-PTBAM) micelles were reported by Yuan et al. in 2012 (Figure 1.12) [128]. A two-step synthetic process was adopted to prepare the triblock copolymers, where the PEO-b-PCL diblock copolymer was first synthesized by organocatalyzed ROP and then chain-extended through the polymerization of TBAM via atom transfer radical polymerization (ATRP). The hydrophobic PCL block was incorporated to drive self-assembly and impart biodegradability, whereas PEO was thought to provide better biocompatibility and colloidal stability. In contrast to most studies that relied on quaternization for antimicrobial activity, the micelles reported in this study were inherently antibacterial and membrane-active through the secondary amine-possessing PTBAM component, which is cationic under physiological conditions. The use of PTBAM could potentially circumvent the significant hemolytic effects of quaternary ammonium-based antimicrobials [143]. The most potent polymer had the highest PTBAM content, with MICs of 0.19 mM and 0.06 mM against *E. coli* and *S. aureus*, respectively. Further, the MICs of the best-performing polymer were found to be at least 10 times greater than its CMC (5.5 µM), which agreed with previous studies by Yang and co-workers that demonstrated self-assembly as a prerequisite for antimicrobial efficacy.

![Figure 1.12. Illustration of the aqueous self-assembly of PEO-b-PCL-b-PTBAM copolymer into micelles, which were postulated to interact with bacterial membranes through electrostatic interactions [128].](image)

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Biodegradable core-shell nanoparticles that were self-assembled from poly(ethylene glycol) (PEG)-functionalized amphiphilic polypeptides were reported by Costanza et al. [129]. NCA-ROP initiated by PEG-NH₂ was used to synthesize poly(Z-L-lysine) (PZLL) as
the first block and a statistical copolymer of PZLL and poly(phenylalanine) (PZLL-stat-PPhe) as the second block (Figure 1.13). Self-assembly in water formed 50-200 nm micelles with a hydrophobic core and a PEG-containing hydrophilic shell. In general, the nanoparticles were more active against the Gram-positive strains, where activity was attributed to membrane disruption as demonstrated through fluorescence and scanning electron microscopy (SEM). Nanoparticle biocompatibility was evaluated in terms of HC$_{50}$ values. The most biocompatible nanoparticles were those that were purely hydrophilic (no PPhe) with HC$_{50}$ values that were greater than 50 µM; however they were only effective against the Gram-positive species. On the other hand, the amphiphilic nanoparticles with 50-60% hydrophobic content exhibited broad-spectrum efficacy against Gram-positive and Gram-negative bacteria, including multidrug-resistant (MDR) species, with MICs that ranged from 5 to 92 µM depending on polymer and bacteria types. The high hemolytic activities (i.e., HC$_{50}$ 5-6 µM) of these nanoparticles, however, rendered them non-selective against bacteria. The results from this study highlighted the importance of balancing polymer hydrophilicity and hydrophobicity to modulate antimicrobial efficacy and biocompatibility.

![Figure 1.13. Synthetic strategy for the PEG-poly(amino acid) copolymers [129]. Copyright 2014. Reproduced with permission from the Royal Society of Chemistry.](image)

An interesting approach to modulate antimicrobial activity and biocompatibility was employed by Wan et al, where mixed micelles were fabricated from the cross-assembly of poly(quaternary ammonium)s (PQAs) in the form of quaternized PDMAEMA (qPDMAEMA) and poly[poly(ethylene glycol) methyl ether methacrylate] (PPEG) (Figure 1.14) [130].
Despite being a well-known antibacterial agent, PQAs, similar to many polycations, have limited therapeutic utility due to their high toxicity towards mammalian cells [144]. Hence, the addition of PPEG which is non-ionic was expected to improve the biocompatibility of the polymer construct, ideally without compromising the potency of the PQAs against bacterial cells. As expected, the PQA homopolymers synthesized as controls exhibited efficacy against \textit{S. aureus} (MIC 1.25-40 µg/mL) and \textit{E. coli} (MIC 10-160 µg/mL). Following the addition of PPEG to form PPEG/PQA mixed micelles, the resulting MICs were affected to different extents depending on the alkyl chain lengths of the PQAs. Noteworthy, a greater reduction in activity against \textit{E. coli} (up to 8-fold) occurred when the PQAs in the mixed micelles had shorter alkyl chains. The authors demonstrated that the PPEG chains shielded the cationic charges of the PQAs, thereby reducing polymer-membrane interactions. Despite this, mixed micelles composed of PPEG and PQAs quaternized by 1-bromooctane displayed low MICs of 39.1 and 19.5 µg/mL against \textit{E. coli} and \textit{S. aureus}, respectively. While the shielding of cationic PQAs by the PPEG chains led to reduced polymer-bacteria activity as compared to PQAs alone, the authors successfully showed that this shielding effect was able to reduce the toxicity of PQAs towards mammalian cells. As such, the treatment of mice fibroblast L929 cells by PPEG/PQA mixed micelles at 100 µg/mL for 72 h resulted in only a marginal loss in cell viability (<10%). This was in stark contrast to the cases where PQA homopolymers were administered, in which more than 60% loss in cell viability was observed at the same concentration.
Chitosan and its derivatives are often used to confer antimicrobial efficacy and biocompatibility to synthetic polymers [27]. However, the poor solubility of chitosan in conditions other than a low pH environment limits its applicability in biomedical applications [145, 146]. In a study conducted by Lin et al., chitosan was grafted with poly(methacryloyloxyethyl trimethylammonium chloride) (PDMC) with DPs ranging from 50 to 400 via single electron transfer living radical polymerization (SET-LRP) in an ionic liquid system [131]. The PDMC-functionalized chitosan displayed self-assembly behaviour into monodispersed, spherical micelles (120-200 nm in diameter) in acetone. The PDMC-grafted chitosan was able to inhibit the growth of *E. coli* by reducing its rate and extent of growth more effectively compared to unmodified chitosan, although quantitative growth inhibition was not attainable within the timeframe of the experiments (24 h). The authors postulated that an increase in positive charge density was achieved through the addition of quaternary ammonium groups found on PDMC, possibly resulting in improved adsorption of the polymer on bacterial cell surfaces. However, the role of self-assembly, if any, in influencing the biological activity of chitosan was not clearly elucidated in this study.
Harnessing the potential of macromolecular assemblies to incorporate multiple functionalities, Li et al. successfully fabricated novel polyion complex (PIC) micelles capable of both (fluorometric) detection and inhibition of bacteria, formed through the interactions between negatively-charged tetraphenylethylene (TPE) sulfonate derivatives and neutral-cationic PEO-\(b\)-qPDMAEMA copolymers (Figure 1.15) [132]. The cationic moieties were expected to be shielded within the micellar cores by the anionic sulfonic acid functionalities in an aqueous environment. When placed in a bacteria-containing medium, the authors postulated that more favorable ionic interactions between bacterial surfaces and qPDMAEMA would lead to micellar disassembly. In fact, the nanostructure disassembly upon bacteria contact resulted in the bacteria sensing capability of these micelles, which was characterized by a decrease in TPE fluorescence intensity due to the loss of aggregation-induced emission (AIE) following the release of free TPE upon micelle disintegration. In addition to their ability to detect \(E\). \(coli\), the PIC micelles were found to be bactericidal through membrane disruption, with the most potent variant registering an MIC of 19.7 \(\mu\)g/mL against \(E\). \(coli\). However, a similar level of efficacy was not shown against \(S\). \(aureus\). When tested for its hemolytic activity, the micelle with the highest antimicrobial activity demonstrated negligible (<2%) hemolysis at a high concentration of 400 \(\mu\)g/mL (20 times greater than its MIC).

Figure 1.15. Synthesis of polyion complex (PIC) micelles from TPE sulfonate derivatives (BSTPE or TSTPE) and PEO-\(b\)-qPDMAEMA block copolymers [132]. Copyright 2014. Reproduced with permission from Elsevier Ltd.

Another example of multifunctional micelles was demonstrated by Luo and co-workers in the form of poly(caprolactone)-poly(quaternary ammonium salt) (PCL-PQA)-based micelles which possessed antibacterial and drug loading properties [133]. This study was aimed at exploring the possibility of exploiting the synergistic effects of inherently antimicrobial polymers and conventional antimicrobial agents to produce hybrid materials with enhanced...
antimicrobial properties. Linear and 3- and 6-arm star copolymers of PCL and PDMAEMA, quaternized with methyl chloroacetate, were shown to self-assemble into spherical micelles with PDMAEMA forming the corona and PCL located within the core. All micelles formed, either from linear or star copolymers, were able to encapsulate an antimicrobial drug, triclosan, in the core and demonstrate two-phase drug release profiles characterized by an initial burst release followed by a slower and more sustained release process. At a polymer concentration of 0.1 mg/mL, all blank micelles (containing no drug) exhibited bactericidal activities to varying extents, with the micelles formed from the 6-arm star copolymer displaying the highest potency (*i.e.*, a 4-fold reduction in *E. coli* colony-forming units, CFU). After the loading of triclosan into the core, enhanced activity against *E. coli* was observed, with near-quantitative (99%) killing reported for the 3- and 6-arm star copolymer-based micelles. Using a standard broth microdilution assay to monitor bacterial growth inhibition, only the 6-arm star copolymeric micelles that were loaded with triclosan were able to cause quantitative growth inhibition, while the inhibitory activities of the other micelle types were insufficient to prevent bacterial re-growth within the incubation period. In general, the authors concluded that star-shaped copolymeric micelles were more effective than micelles formed from the linear copolymers, which was likely due to a higher charge density provided by the star copolymers.

While most studies focused on the fabrication of spherical micelles, the potential of rod-shaped micelles as effective antimicrobials was demonstrated by Hedrick and co-workers [134, 135]. In 2012, an interesting study was published to elucidate the shape effects of a self-assembled polycarbonate-based system by comparing the biological activities of spherical and rod-like micelles with similar size and charge density (Figure 1.16) [134]. The nanostructures were constructed from triblock copolymers synthesized *via* organocatalyzed ROP and initiated by a terephthalamide-bisurea bifunctional initiator which forms a rigid, assembly-directing core. The inner block consisted of a polylactide (L-, D-, or rac-PLA), whereas the hydrophilic outer block was composed of quaternized poly[2-(3-bromopropyl)oxycarbonyl-2-methyl trimethylene carbonate] (PMTC-BP) which conferred the resulting nanostructures with antimicrobial activity. Direct self-assembly in an aqueous environment was shown, where spherical or rod-shaped nanostructures were obtained depending on the chemical structure of the core. The addition of a methylene spacer in the core resulted in rod-shaped supramolecular structures (*ca.* 10 nm in diameter and aspect ratios ≥ 10), instead of the typical spherical assemblies (*ca.* 20.4 nm in diameter) that were
obtained when the spacer was absent. It was noted that the role of lactide stereochemistry on nanostructure size and shape was minimal. The assemblies, regardless of shape and lactide stereochemistry, were non-hemolytic even at high concentrations of 1 to 5 mg/mL. Both spherical and rod-shaped nanostructures exhibited broad spectrum efficacy against bacteria such as *S. aureus*, MRSA, vancomycin-resistant *Enterococci* (VRE), *E. coli*, and fungal species *C. neoformans*. The MICs ranged from 20 to 150 µg/mL and 20 to 100 µg/mL for the spherical and rod-like assemblies, respectively, with poly(L-lactide) inner blocks. Interestingly, while the spherical nanoparticles were inactive against *C. albicans* (MIC > 500 µg/mL), the poly(L-lactide)-based rods were able to cause quantitative fungal growth inhibition at 75 µg/mL. Through TEM and confocal microscopy imaging, the nanostructures were shown to kill bacteria and fungus through membrane disruption. As fungal species such as *C. albicans* are more difficult to kill due to their multi-layered, thicker and less negatively charged cell walls, the results reported in this study are significant and represents advancement in the development of antifungal drugs. However, it should be noted that although the MICs recorded against most species were greater than the polymer CMCs, antimicrobial action against certain microbial species (such as *C. neoformans*) was resulted at polymer concentrations below the CMCs.

**Figure 1.16.** The formation of spherical and rod-like assemblies from terephthalamide-polylactide (PLA)-polycarbonate triblock copolymers, and their antimicrobial properties [134]. Copyright 2012. Reproduced with permission from the American Chemical Society.

Encouraged by the antifungal efficacy of rod-shaped polymer assemblies in the earlier study [134], Hedrick and co-workers developed a novel class of cationic, amphipathic and low-molecular weight compounds, capable of forming antifungal high-aspect ratio supramolecular assemblies with low propensity for resistance development [135]. Despite the fact that the assemblies were not constructed from polymers, the self-assembled nanostructures manifested polymer-like properties, such as a glass transition temperature ($T_g$),
and the formation of fiber-like morphologies in water. The antifungal agents were based on terephthalimide-bisurea compounds bearing different spacer groups between the urea and cationic charge, and the formation of nanorods was induced and stabilized by hydrophobic interactions and amide-amide hydrogen bonding. Depending on the type of spacer incorporated, nanofibers of varying rigidity and sizes were fabricated. In this study, the MICs of all compounds against *C. albicans* and *C. neoformans* were found to be greater than their CMCs, indicating that the antifungal agents were active as supramolecular assemblies. The authors further postulated that the formation of these high aspect ratio assemblies allowed for more efficient fungal cell wall and membrane insertion, which was subsequently validated through electron microscopy in the form of observable membrane damage and cell lysis following treatment. Rigid nanofibers assembled from terephthalimide bisurea with benzyl amine spacer groups demonstrated the highest efficacy against *C. albicans* and *C. neoformans*, with an MIC of 31.2 µg/mL against both species. Further, the nanorods were shown to be effective against *C. neoformans* resistant to fluconazole, a common antifungal drug. Noteworthy, a representative nanorod variant displayed *in vitro* and *in vivo* efficacy against *C. albicans* biofilm, which suggested the potential of these nanostructures in combating fungal infections, such as those associated with medical implants and fungal keratitis. This was supported by the general lack of significant hemolytic effects and mammalian cell toxicity within the therapeutic concentration range, as well as *in vivo* biocompatibility demonstrated through a mice cornea model.

### 1.3.3.1.2 Polymeric Vesicles

Polymeric vesicles, also known as polymersomes, are spherical polymeric capsules with an inner hollow compartment confined by a bilayered membrane which is composed of amphiphilic block copolymers [147, 148]. Acting as simple mimics of biological cells, polymeric vesicles provide three compartmentalized regions available for functionalization, namely the inner hydrophilic cavity, the polymer shell, and the periphery in contact with the environment [149]. Compared to typical micelles that possess an inner hydrophobic core and a hydrophilic shell, the complex morphology of polymeric vesicles allows for more enhanced compartmentalization and increased versatility in functionalization [150], which have proven to be useful in applications such as drug delivery [151-153]. For example, polymeric vesicles are capable of encapsulating both hydrophilic and hydrophobic compounds within their cavities and membranes, respectively [148]. In the area of antimicrobial research, several investigations have been performed on polymeric vesicles to explore their potential as
antimicrobials, although vesicles are not as extensively studied as polymeric micelles. More importantly, researchers have attempted to utilize the compartmentalization offered by polymersomes, coupled with the judicious selection of polymer building blocks, to develop multifunctional and inherently antimicrobial nanostructures.

The solvent-switch method is conventionally used to prepare polymeric vesicles, where the copolymers are first solubilized in an organic solvent, which is then gradually replaced with water to induce vesicle formation [154]. This strategy was adopted by Du and co-workers to prepare well-defined and water-dispersible poly(methyl methacrylate)-b-PTBAM (PMMA-b-PTBAM) vesicles [155]. The merging of PMMA, which is one of the most widely used polymeric materials, and the antimicrobial PTBAM in a single construct was intended to impart antibacterial properties to the biologically inert PMMA, which may further expand the applicability of PMMA in the biomedical field. As PTBAM is amphiphilic, the hydrophilic portion of PTBAM formed the vesicle corona while its hydrophobic portion together with PMMA constituted the membrane. The vesicles were approximately 250 nm in diameter with a membrane thickness of 16 nm. Efficacy against both *E. coli* and *S. aureus* was demonstrated with MICs of 0.505 and 0.252 mM, respectively, where a higher activity was shown against the Gram-positive species.

Recognizing the limitations of the ‘solvent-switch’ method due to the use of organic solvents, Du and co-workers devised an organic solvent-free approach for the fabrication of water-soluble and antibacterial polymeric vesicles [156, 157]. Amphiphilic block copolymers of poly[2-(2-methoxyethoxy)ethyl methacrylate] (PMEO<sub>2</sub>MA) and PTBAM were synthesized via ATRP and allowed to self-assemble into ca. 240 nm vesicles in low-pH aqueous buffer by heating to 37 °C (Figure 1.17) [156]. Using the spread plate method where the bacterial solution was incubated for 2 h with vesicle solution that was spread on a glass sheet, near-quantitative (close to 3-log or 99.9%) reductions in *E. coli* and *S. aureus* cell counts were observed at a polymer concentration of 0.25 mg/mL. In contrast, the bulk copolymer (in the non-assembled state) was only able to reduce the bacterial cell counts by half at the same concentration. It was hypothesized that vesicle formation led to an increased local concentration of positive charges, resulting in stronger polymer-bacteria affinity compared to unassembled polymer chains. In a subsequent study conducted by the same group, 30 to 40 nm polymeric vesicles based on triblock copolymers, PEO-b-poly(2-diethylaminoethyl methacrylate) (PDEA)-b-PTBAM, prepared via pH switching from acidic to neutral was reported [157]. Self-assembly by adjusting the pH of the medium was possible
due to the pH-responsive nature of PDEA, where the polymer becomes hydrophobic in neutral or basic environments. The best-performing polymersome in terms of antibacterial ability was determined to be the variant with the highest PTBAM content (i.e., PEO\textsubscript{43-}\textit{b}-PDEA\textsubscript{20-}\textit{b}-PTBAM\textsubscript{30}), suggesting that a higher positive charge density on the polymer would promote more efficient interactions with bacterial cell membranes. Interestingly, the PEO\textsubscript{43-}\textit{b}-PDEA\textsubscript{20-}\textit{b}-PTBAM\textsubscript{30}-based polymersome was more effective against \textit{E. coli} (MIC 0.15 mM) than \textit{S. aureus} (MIC 0.6 mM), different from the trend shown in their previously reported PTBAM-based polymeric vesicle system [155].

![Figure 1.17. The formation of antibacterial vesicles from block copolymers of PMEO\textsubscript{2}MA and PTBAM, and their postulated antibacterial mechanism [156]. Copyright 2013. Reproduced with permission from the Royal Society of Chemistry.](image)

One of the earliest studies on polymeric vesicles with combined antibacterial and drug delivery capabilities was reported by Zhu \textit{et al.} in 2013 [158]. An interesting class of antibacterial vesicles in the form of high-genus, perforated vesicles was developed from PMEO\textsubscript{2}MA-\textit{b}-PTBAM copolymers synthesized \textit{via} ATRP. A two-stage assembly process aided by the phase transition of the thermo-responsive PMEO\textsubscript{2}MA block was employed, where an initial solvent-switching step yielded branched cylinders, which transited to a high-genus vesicular morphology following heating to 37 \degree C. The resulting vesicles had an average overall diameter of \textit{ca.} 400 nm with \textit{ca.} 20 nm pores on the surface. With MICs of 0.7 and 0.35 mM against \textit{E. coli} and \textit{S. aureus}, respectively, and quantitative bacterial cell killing at 0.25 mg/mL, the high-genus vesicles exhibited improved efficacy compared to the bulk copolymer and simple vesicles based on the same polymer system. This was attributed
to the more complex hierarchical structure of the high-genus vesicles synthesized in this study. When tested against mammalian cells, the vesicles demonstrated low toxicity (defined as >80% cell viability) against human HCCLM3 liver cancer cells and L02 liver cells up to 0.5 mg/mL, as well as excellent blood compatibility (HC_{50} 4.7 mg/mL). However, it should be noted that a direct comparison of the antibacterial and mammalian cell studies to yield selectivity indices was not possible, as the effective concentrations were reported in different units. Additionally, the authors showed that the high-genus vesicles were able to encapsulate doxorubicin (a hydrophobic anticancer drug) and manifest an acid-accelerated drug release profile, which could be useful for targeted drug delivery to tumor sites. As such, the authors postulated that these complex nanoparticles have potential to be used in applications where simultaneous antibacterial and anticancer therapies are required.

In line with the ultimate goal of developing drug-carrying antibacterial nanostructures, Zhou et al. reported the synthesis of polypeptide- and chitosan-based nanocapsules, where statistical copolymers of poly(L-lysine) (PLL) and PPhe from NCA-ROP were statistically grafted onto an acid-functionalized chitosan backbone (Figure 1.18) [159]. Subsequently, half of the chitosan carboxyl groups were esterified, whereas the remaining acid groups were left unmodified for drug conjugation. The self-assembled nanostructures were hypothesized to possess a hydrophilic corona and a complex membrane which was composed of both hydrophobic and entrapped hydrophilic moieties due to hydrogen bonding and steric effects. The authors were unable to compare the efficacy of the nanocapsules with the non-assembled form as the critical concentration for nanocapsule formation was less than their MIC. Hence, comparisons could only be made with the individual polypeptide chains before grafting to chitosan as well as nanocapsules where the chitosan was not esterified. The formation of these vesicle-like assemblies resulted in a 2-fold improvement in efficacy against *E. coli* and *S. aureus* (MIC 16 µg/mL) compared to linear polypeptides PLL-stat-PPhe, which are the effective antibacterial component of the nanocapsules. Besides the charge localization effects brought about by self-assembly, antibacterial activity was also promoted by the esterification of carboxylic acid groups on the chitosan backbone, as this reduced the overall negative charge of the construct. Interestingly, compared to linear PLL-stat-PPhe, the nanocapsules showed higher hemo-compatibility (HC_{50} 700 µg/mL; HC_{50}/MIC 44) and cyto-compatibility with human HCCLM3 liver cancer cells (*i.e.*, 70% cell viability after 72 h incubation at 100 µg/mL). Additionally, the encapsulation and subsequent enzyme-triggered release of both anticancer and antiepileptic drugs were demonstrated for these vesicle-like nanostructures.
Figure 1.18. Schematic illustration of the self-assembly of polypeptide-grafted chitosan-based nanocapsules, and its ability for drug release upon enzymatic (protease) degradation [159]. Copyright 2013. Reproduced with permission from the American Chemical Society.

In a follow-up study by Wang et al., a fully biodegradable, peptide-based vesicle platform was developed with a lower critical vesiculation concentration (CVC) and demonstrated stability in animal serum [160]. Statistical amphiphilic polypeptide copolymers, PLL-stat-PPhe, and the hydrophobic PCL were synthesized by NCA-ROP and organocatalyzed ROP, respectively. The polypeptides and PCL were then conjugated, which was followed by the deprotection of PLL and post-functionalization with folic acid moieties for potential cancer cell targeting applications. Using the ‘solvent-switch’ method, vesicles with a $D_H$ of 301 nm and a CVC of ca. 13 µg/mL were obtained where PLL-stat-PPhe and PCL constituted the corona and membrane, respectively. Compared to the free PLL-stat-PPhe copolymer chains which registered an MIC of 32 µg/mL against E. coli and S. aureus, the vesicles were more active with an MIC of 16 µg/mL against both bacterial species. This enhancement in activity was attributed to vesiculation and possible synergistic interactions between PCL and the polypeptides. However, the reader should be made aware that the definition of MIC adopted in this study was based on a more than 50% inhibition in bacterial growth relative to the untreated control, which is different from the more widely used convention where MIC is taken as the concentration where quantitative growth inhibition (characterized by the absence of visible growth) is resulted [85]. In terms of the toxicity of these vesicles towards normal liver cells L02, only marginal (<20%) decrease in cell metabolic activities was observed after 72 h when treated with a wide range of vesicle concentrations (62 to 250 µg/mL), suggesting
that these nanostructures had relatively low toxicity. A preliminary antitumor activity study was also conducted, where the vesicles were loaded with doxorubicin and shown to be capable of killing liver cancer cells through the released doxorubicin.

In addition to the potential applications of antibacterial polymeric vesicles in the field of nanomedicine, Geng et al. demonstrated their possible use in water remediation, where effective bacterial growth inhibition and the eradication of carcinogenic organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs), are often required in parallel [161]. Vesicles were constructed from a statistical copolymer, poly[2-hydroxy-3-(naphthalene-1-ylamino)propyl methacrylate] (PHNA)-stat- PTBAM, synthesized using reverse addition-fragmentation chain-transfer (RAFT) polymerization. The PTBAM component imparted antibacterial properties to the vesicles, while PHNA is capable of sequestering PAHs through π-π stacking. The vesicles were able to reduce bacterial growth by half at \( \text{ca. 26.2 and 15.5 } \mu \text{M} \) against \( E. coli \) and \( S. aureus \), respectively. In a simulated polluted water environment containing pyrene (as a PAH mimic) and \( E. coli \), complete eradication of bacteria and pyrene was observed after vesicle treatment.

1.3.3.2 Star Polymeric Nanoparticles

Despite the versatility of self-assembly and the potential shown by self-assembled polymeric nanoparticles in the antimicrobial field, the reversible and dynamic nature of self-assembly renders the fabricated nanostructures prone to disassembly and possibly not sufficiently robust for practical applications [115]. In contrast to self-assembled polymeric nanoparticles, star polymeric nanoparticles are covalently-bonded unimolecular nanostructures with a branched architecture consisting of several linear chains linked to a central core, thereby forming 3D globular structures [96]. Such nanoparticles have become of interest due to their unique structural properties. It is possible to synthesize high molecular weight star-shaped polymeric nanoparticles while still retaining a solubility and viscosity similar to that of low molecular weight linear or branched polymers [162]. Other attractive features of star polymers include their encapsulation capabilities [96, 163-165], and a vast range of internal, peripheral and compartmentalized functionalities [96]. The reader is referred to a comprehensive review published by Qiao and co-workers on the synthesis, characterization, properties and applications of star polymers [166].

Recently, the applicability of star polymers in the field of nanomedicine have been reviewed by Li and co-workers [167], with applications in targeted drug delivery [168-172],
gene therapy [173-176], bioimaging [171, 177, 178], and tissue engineering [179] being explored by numerous research groups. However, in comparison to the other biomedical applications, the design and development of star polymeric nanoparticles with inherent antimicrobial properties have not been studied extensively, and the research efforts thus far will be highlighted in this section.

Early studies in this area were aimed at covalently linking bactericidal compounds such as antibiotics and AMPs to a polymeric scaffold, resulting in antibacterial nanostructures with a star-like architecture. Arimoto et al. synthesized a multivalent polymer of the antibiotic vancomycin via ruthenium-catalyzed ring-opening metathesis polymerization (ROMP) by conjugating vancomycin to a metathesis-active norbonene unit [180]. This was done in an attempt to enhance the potency of vancomycin in response to the rise of vancomycin-resistant Enterococci (VRE). While the branched polymer was found to have broad molecular weight distributions, an 8 to 60-fold enhancement in potency against VREs was observed compared to unmodified vancomycin. Taking inspiration from antimicrobial dendrimeric peptides which comprise of a multivalent core with an array of branching peptides tethered to it [181], Kallenbach and co-workers fabricated multivalent AMPs using a non-antimicrobial poly(maleic anhydride) (PMA) chain as a scaffold to link antimicrobial tetrapeptides (either RRWW-NH₂ or RWRW-NH₂) into a branched-like nanostructure [182]. The tethered AMPs demonstrated a 10-fold improvement in MIC₅₀ (i.e., one-half of the MIC) against E. coli and B. subtilis, which was hypothesized to be due to the increased in local density and synergy of the linked peptides. However, the tethering of these AMPs to the PMA scaffold did not improve the selectivity of the peptides, as the aforementioned improvement in growth inhibitory activity after tethering was accompanied by an increase in hemolytic activity. While further investigations and optimizations are warranted for these early studies, they formed the groundwork for subsequent work by demonstrating that antimicrobial efficacy could be enhanced with a multivalent display of microbicidal components achieved through a star-like architecture.

Extending from this concept, Liu et al. synthesized star-shaped molecular brushes composed of qPDMAEMA via ATRP using cyclotriphosphazene initiators [183]. The effect of side chain hydrophobicity was evaluated by using quaternizing agents with different alkyl lengths, and increased activity against E. coli was observed with longer hydrophobic alkyl side chains. Comb-shaped qPDMAEMA brushes were also synthesized but were found to be inferior in antibacterial efficacy compared to the star polymers. It was postulated that while a
more condensed positive charge density could lead to better antibacterial activity, an overly dense grafted brush structure such as in the case of the comb-shaped polymers could make the active polymeric components less accessible by bacterial cells. The best-performing star polymer had a moderate MIC of 250 µg/mL against *E. coli*; however, a comparison of antimicrobial activity with linear qPDMAEMA and mammalian cell compatibility studies were not conducted.

Using a similar core-first approach, star-shaped polymers with poly(styrene)-*b*-poly(4-vinyl-*N*-methylpyridinium iodide) (PS-*b*-P4VMP) arms were prepared by anionic polymerization, as reported by Tiller and co-workers [184]. Poly(4-vinyl-*N*-alkylpyridinium)-based polymers, which contain quaternary ammonium groups, have attracted great interest as antimicrobial materials because of their superior antibacterial efficacy against both Gram-positive and Gram-negative bacteria [185]. While the primary goal of the study was to develop semi-permanent antimicrobial coatings, the star polymers were found to be active against *S. aureus* in solution, with MICs ranging from 78 to 156 µg/mL. Contrary to the earlier studies on potency enhancement through multivalent nanostructures, the MICs of the star polymers were found to be similar to that of linear P4VMP, which suggested that the star architecture and the additional PS block had no effect on the antimicrobial properties of the final construct.

By taking advantage of hierarchical functionalities spanning from the periphery along the arms and to the core afforded by the star architecture, recent studies on star polymeric nanoparticles in the antimicrobial field tend to focus on the development of multifunctional polymer platform technologies, by imparting diagnostic [186], antiviral [187], or targeting [94, 188] functionalities in addition to the inherent antibacterial properties of the star nanoparticles. A bacterial detection and inhibition system based on four-arm star polymers with a tetraphenylethylene (TPE) core and PDMAEMA-*co*-butyl methacrylate-*co*-Gd, P(DMAEMA-*co*-BMA-*co*-Gd) arms was developed by Li et al. through a combination of ATRP and post-modification reactions (Figure 1.19) [186]. Bacterial inhibition capabilities were provided by the cationic and amphiphilic star arms, while the *T*<sub>1</sub>-type magnetic resonance (MR) imaging contrast agent, DOTA-Gd, enabled bacterial detection via fluorescence and MR imaging. In terms of antimicrobial performance, the star polymer with the most abundant hydrophobic (BMA) moieties, TPE-*star*-P(DMAEMA<sub>0.79</sub>-*co*-BMA<sub>0.15</sub>-*co*-Gd<sub>0.06</sub>)<sub>24</sub>, was found to be the most effective against *E. coli*, *P. aeruginosa* and *S. aureus* with higher efficacy observed against the Gram-negative species over the Gram-positive species.
tested (i.e., MICs of 5.5 µg/mL and 0.12 µg/mL against *E. coli* and *P. aeruginosa* compared to an MIC of 30 µg/mL against *S. aureus*). As visualized through SEM, treatment of *E. coli* with the star polymer resulted in outer and inner membrane deformations. However, it is noteworthy that the most antimicrobial star polymer was found to possess the highest hemolytic activity with a HC$_{10}$ value (i.e., the polymer concentration required to cause 10% hemolysis) of 0.01 µg/mL, therefore rendering the polymer non-selective towards bacterial cells. In order to mitigate the lack of biocompatibility, quaternization of PDMAEMA on the star arms was performed and resulted in significantly lowered haemolytic activity by more than 70,000-fold for the star polymer with the highest antimicrobial efficacy, although a slight reduction in inhibitory activity (2-fold against *E. coli*) was observed.

**Figure 1.19.** Schematic illustration of the structure of TPE$_{core}$P(qDMAEMA-co-BMA-co-Gd)$_{arm}$ star polymers [186]. Copyright 2014. Reproduced with permission from John Wiley & Sons Inc.

With the aim of developing nanoparticles that are both antibacterial and antiviral, Xiao and co-workers fabricated 5 and 8-arm star polymers by randomly polymerizing poly(hexamethylene guanidine hydrochloride) (PHMG) macromonomers and acrylamide (AM) via ATRP initiated by β-cyclodextrin [187]. Polymeric guanidines, such as PHMG, are well-known membrane-disrupting antimicrobials that are effective against a range of Gram-positive and Gram-negative bacteria, fungi, and yeasts [189]. The 8-arm star variant with an AM:PHMG molar ratio of 20:3 exhibited the highest efficacy against *E. coli* (MIC 0.78 µg/mL). When compared to the substantially lower activity of PHMG homopolymer against *E. coli* (MIC 7.8 µg/mL), the authors postulated that the star architecture was responsible for enhancing the antimicrobial performance of PHMG. However, while PHMG was identified...
as the antibacterial component, the role of AM in the star polymer system was not elucidated. The authors further demonstrated that the best-performing star polymer was non-toxic towards human embryonic kidney cells (HEK 293) at or below 50 µg/mL.

While earlier studies have shown that the proper design of star polymeric nanoparticles could facilitate excellent antimicrobial activity, the toxic, off-target side-effects of some of these nanoparticles, such as towards healthy mammalian cells or probiotic bacteria, are a major concern. In their efforts to achieve targeted antimicrobial therapy, several research groups have focused on the attachment of targeting ligands, such as sugar-based moieties, on antibacterial star polymers [94, 188]. Using a multifunctional $\beta$-cyclodextrin-based macroinitiator, Yang and co-workers reported the synthesis of star-shaped polycarbonates by metal-free organocatalytic ROP of benzyl chloride (BnCl) and mannose-functionalized cyclic carbonate monomers (Figure 1.20) [94]. The star arms were synthesized as either block or random copolymers, and further subjected to deprotection of the mannose protecting groups and quaternization of BnCl with various $N,N$-dimethylalkylamines. It was envisioned that the mannose functional groups would facilitate the targeting of mannose receptors expressed on the surface of macrophages (i.e., a type of immune cells), leading to the internalization of the polymers and subsequent killing of any intracellular pathogenic bacteria. The mannose-functionalized star polymers were shown to be membrane-lytic and capable of eradicating both Gram-positive ($S. aureus$) and Gram-negative ($E. coli$ and $P. aeruginosa$) bacterial species as unimolecular nanostructures, although preferential activity was shown towards the Gram-positive species (MIC 0.09-2.78 µM). While mannose functionalization reduced the activity of the star polymers against the Gram-negative species, potency against $S. aureus$ was still retained. A linear ‘one arm’ equivalent was also synthesized, but its antibacterial efficacy was found to be inferior to the corresponding star polymer. By systematically varying the compositions of the star polycarbonates, it was demonstrated that increasing the arm length (by increasing the DP of the BnCl carbonate monomer units) and quaternizing with more hydrophobic agents led to improved antimicrobial activity. Interestingly, an increase in polymer hydrophobicity also led to increased hemolytic activities, while the introduction of more mannose groups reduced the red blood cell lytic properties of the polymers. The star nanoparticles with the highest bacteria over red blood cell selectivity were found to be those that were mannose-functionalized, possessed a high DP of BnCl units, and quaternized with the least hydrophobic group. This highlighted the importance of balancing hydrophilicity and hydrophobicity as well as the degree of mannose functionalization on the
star nanostructure for optimal antimicrobial activity and selectivity. Additionally, the authors demonstrated the ability of a model star polycarbonate in reducing the number of intracellular *Mycobacterium bovis* BCG (a Gram-positive bacterium) in human monocyctic THP-1 cells via the targeting of mannose receptors present on the THP-1 cell surface. However, the cytotoxicity of the star polymer towards THP-1 cells at concentrations above 0.2 µM (which fall within the MIC range of the polymers against certain bacteria) would hinder its clinical use.

![Chemical structure of cationic and mannose (man)-functionalized star-shaped polycarbonates](image)

*Figure 1.20.* Chemical structure of cationic and mannose (man)-functionalized star-shaped polycarbonates [94].

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In order to modulate the antimicrobial activity and mammalian cell compatibility of star polymers, Wong *et al.* constructed a series of 14-26 nm, glucosamine-functionalized star polymers with 12 to 19 arms using an arm-first approach [188]. Poly(L-lysine) (PLL) and poly(glucosamine) (PGSA)-based arms were synthesized and then converged to a central cross-linked core using a combination of RAFT polymerization, NCA-ROP, and click chemistry. PLL was designed to be the antimicrobial component, while the glycopolymer was introduced to reduce mammalian cell toxicity and improve the infiltration of the star polymers through the peptidoglycan (PG) layer of bacterial cells. Despite moderate star conversions (60-65%) and the absence of hydrophobic groups, the miktoarm star polymers exhibited preferential activity against Gram-positive bacteria over the Gram-negative species, and were effective against a range of Gram-positive bacteria including MRSA and VRE. Notably, the PLL-PGSA star with 25% GSA content (S-GSA 25) was found to be the most potent, with MICs ranging from 16 to 64 µg/mL against *S. aureus*, MRSA, *E. faecalis*, and VRE. All star polymers synthesized were non-hemolytic even at a high concentration of 10 mg/mL, which was likely to be attributed to the lack of hydrophobic groups. While most polycations, such as PLL, are known for their high toxicity towards eukaryotic cells [190],...
the incorporation of PGSA mitigated the toxicity of the PLL-PGSA stars against human aortic smooth muscle cells, resulting in high cell viability (>80%) for all GSA-containing stars at 100 µg/mL. The authors further demonstrated that the excellent biocompatibility of the stars was afforded by the star architecture, as a blend of linear PLL and PGSA homopolymers could not provide the same biocompatibility as observed for the heteroarm PLL-PGSA stars. It was hypothesized that when locked in a star-shaped nanoparticle form, PLL arms were shielded by the PGSA-based chains, thereby reducing the likelihood of non-specific interactions between PLL and mammalian cell surfaces. With negligible hemolytic activity and an IC$_{50}$ of 337 µg/mL, S-GSA 25 was shown to possess favorable therapeutic indices and hence outperformed conventional AMPs such as indolicidin and polymyxin B.

Figure 1.21. Illustration of the chemical structure of glucosamine-functionalized star polymers [188]. Copyright 2016. Reproduced with permission from the American Chemical Society.

### 1.3.3.3 Inorganic-Polymer Hybrid Nanoparticles

Inorganic-polymer core-shell nanoparticles are typically composed of a metal- or silica-based core with a polymer shell [191]. Conventionally, in order to expand the applicability of inorganic nanoparticles, such as metal or metal oxide nanoparticles, to suit a wider range of applications, they are often coated with polymer to improve the oxidation stability of the core, reduce particle aggregation in solution, and improve mammalian cell compatibility for bio-applications [191]. In the development of inorganic-polymer hybrid core-shell nanostructures for antimicrobial applications, earlier studies have focused on the use of a biologically inert silica core coated with a layer of bactericidal polymer [192-195]. More recent studies, however, have highlighted the potential of harnessing the synergistic functions of bactericidal metal-based nanoparticles and polymers with direct antimicrobial properties [196, 197].

The following discussion will be divided into two parts, namely antimicrobial silica-polymer core-shell nanoparticles and polymer-coated metal-based nanoparticles.
1.3.3.3.1 Silica-Polymer Core-Shell Nanoparticles

Using vapor deposition polymerization (VDP), the fabrication of silica nanoparticles coated with either PTBAM-co-poly(ethylene glycol dimethacrylate) (PTBAM-co-PEGDMA) or PDMAEMA-co-PEGDMA was reported by Jang and co-workers [192, 193]. PTBAM and PDMAEMA are known microbicidal polymers, whereas EGDMA was used as a cross-linker to improve structural stability and reduce protein fouling on the nanoparticles. It is unclear as to whether the incorporation of EGDMA to the nanoparticle construct would result in the lowering of antimicrobial activity compared to similar nanoparticles with a purely PTBAM or PDMAEMA coating. Using differently sized silica nanoparticles as the core, core-shell nanostructures with average diameters that ranged from 17 to 69 nm and a consistent average polymer shell thickness of 3-4 nm were synthesized. As a result of their large surface areas, the nanoparticles with the smallest size (regardless of the type of polymer coating) were able to eradicate \textit{E. coli} and \textit{S. aureus} quantitatively and at the highest rate with the lowest MIC (<2 mg/mL) compared to the larger nanoparticles and bulk copolymer controls. It is noteworthy that contrary to most studies where additional quaternization of PDMAEMA was required to provide antibacterial efficacy [130, 132, 183], the silica-PDMAEMA-co-PEGDMA core-shell nanoparticles were active without quaternization [193]. The authors hypothesized that the densification of protonated amine groups on the nanoparticle surface afforded by the large surface area was sufficient to promote antibacterial performance. In order to investigate factors affecting the antibacterial activities of amine-functionalized polymer-coated silica nanoparticles, the authors prepared a series of uniform-sized silica nanoparticles coated with different amine-functionalized polymer shells [194]. While the PDMAEMA-, qPDMAEMA-, PTBAM- and polydiallylamine-coated silica nanoparticles produced significant killing against \textit{E. coli} and \textit{S. aureus} at 1 mg/mL, the silica-polypyrrole (PPy) nanoparticles were non-active at the same concentration. As all other polymers contain nitrogen atoms that were protonated under antimicrobial test conditions except for PPy due to extensive lone-pair electron delocalization over the diene [198], the study suggested that the presence of amino groups on a polymer construct did not guarantee antibacterial activity. On the other hand, antibacterial activity was more dependent on the state of the amine groups such that \textit{N}-protonation can be induced.

In a separate study by the same research group, the ability to coat silica nanoparticles with the antibacterial polyrhodanine – known for its ability to kill bacteria by inhibiting bacterial ribonucleic acid (RNA) synthesis – via chemical oxidation polymerization was
demonstrated [195]. When tested against *E. coli* and *S. aureus*, the 15 nm nanoparticles were found to be the most potent, registering MICs of 0.75 and 1 mg/mL, respectively. A similar trend as that reported in their previous studies was observed [192, 193], where the bacterial growth inhibitory activity of the nanoparticles decreased with increasing nanoparticle size. Using a combination of SEM and TEM, the authors postulated that the nanoparticles interacted with bacterial cell membranes, resulting in the loss of membrane integrity and, possibly, DNA damage.

### 1.3.3.3.2 Metal/Metal Oxide-Polymer Core-Shell Nanoparticles

Silver-containing systems, especially silver nanoparticles (AgNPs), have gradually gained recognition as a promising class of microbicidal agents to combat antimicrobial resistance [199]. Harnessing the broad-spectrum antimicrobial properties of AgNPs, Wang *et al.* synthesized a series of polymer-Ag nanocomposites using cationic branched poly(sulfone amine)s (PSAs) as templates [200]. The PSAs were obtained through the reaction of divinylsulfone and 1-(2-aminoethyl)piperazine, which is a trifunctional amine monomer, *via* Michael addition in a dimethylformamide (DMF)-water mixed solvent system. Different branched architectures of PSA were afforded by tuning the volumetric ratio between DMF and water, where a higher water content yielded more hyperbranched units. The PSA-AgNP nanocomposites were formed as Ag\(^+\) ions complexed to the PSAs and underwent *in situ* reduction. Based on a standard disk diffusion assay against *Aspergillus niger* (*i.e.*, a fungal species) at a fixed nanoparticle concentration of 50 mg/mL, the PSA-AgNP nanocomposites were found to be more effective than pure PSAs. An increasing trend in the diameter of the fungal growth inhibition zone was observed with increasing degree of branching. In general, a larger diameter of the growth-free zone surrounding the disk signifies a greater antimicrobial activity of the material contained in the disk. As smaller-sized AgNPs were obtained using more hyperbranched PSAs as templates, the authors postulated that this would yield better surface interactions and penetration ability of the hybrid nanostructures, hence resulting in more efficient antifungal performance. Specifically, the nanocomposite formed using PSA with the highest degree of branching (*i.e.*, 0.41) resulted in more than 80% fungal growth inhibition at a low Ag concentration of 3 µg/mL. While low toxicity against a model eukaryotic cell line (COS-7 cells) was demonstrated by the branched PSAs, the combined toxicity effects of PSAs and AgNPs in the nanocomposite form was not evaluated. This requires further investigation as the high toxicity of AgNPs has been suggested in various publications [201].
Utilizing a similar approach where polymer templates were used to form polymer-stabilized metal nanoparticles, Jia and co-workers prepared core-shell nanoparticles with an AgNP core and qPDMAEMA polymer shells (Figure 1.22) [196]. Linear PDMAEMA was first synthesized using RAFT polymerization and then quaternized with various alkyl bromides. This was followed by the complexation of Ag$^+$ ions with dithioester end groups present on the alkylated polymer and the subsequent reduction of the dithioester to thiols, forming AgNP-qPDMAEMA core-shell nanoparticles. Taking into account the antibacterial activities and cytocompatibility of the core-shell nanoparticles, the nanoparticles with pendant butyl groups were found to possess the highest bacteria selectivity. The growth of *P. aeruginosa* and *S. aureus* was inhibited when nanoparticle concentrations that were greater than 0.4 and 0.2 µg/mL, respectively, were used. Synergistic interactions between AgNPs and qPDMAEMA which resulted in enhanced antibacterial effects were validated as the zones of inhibition for AgNP-qPDMAEMA nanoparticles were significantly larger than those of either AgNP or qPDMAEMA alone. A preliminary mechanistic study suggested that the nanoparticles were able to disrupt bacterial membranes, penetrate into the bacteria and inhibit intracellular enzymatic activity. When evaluated against NIH3T3 fibroblast cells which act as a model mammalian cell line, cell viability remained above 80% even at a concentration which exceeded the MIC by 20-fold. Using a *P. aeruginosa*- and *S. aureus*-induced wound infection model, nanoparticle treatment was shown to promote wound healing after 24 days in both healthy and immunocompromised (i.e. diabetic) rats. Noteworthy, no resistant *P. aeruginosa* or *S. aureus* species was detected against the hybrid nanoparticles.

![Figure 1.22. Synthetic pathway of qPDMAEMA-decorated silver nanoparticles (AgNPs) [196].](image)

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The innate antimicrobial efficacy of gold nanoparticles (AuNPs) remains a widely debated issue. While AuNPs have been recently thought to be at most weakly bactericidal at high concentrations, the ease and versatility of the nanoparticle platform for functionalization (such as with polymers) endow AuNPs with the potential to act as antimicrobial therapeutic agents. Recently, recyclable antibacterials with targeted efficacy against pathogenic E. coli, in the form of 18-22 nm AuNPs with immobilized poly[(2-methacrylamide)glucopyranose] (PMAG) and poly[2-(methacryloyloxy)ethyl trimethylammonium iodide] (PMETAI) (also known as qPDMAEMA), were reported by Yuan et al. The polymers were synthesized by RAFT polymerization and subjected to aminolysis to afford terminal thiol groups for grafting onto AuNPs. PMETAI formed the antibacterial component as it contains quaternary ammonium groups, whereas PMAG is a glycopolymer that could bind with the mannose-binding lectin, FimH, found on the tips of E. coli fimbriae. The target specificity of the nanoparticles was demonstrated by evaluating their efficacy against S. aureus, where a much lower bactericidal effect was observed due to the absence of sugar-binding fimbriae on the Gram-positive bacterial cells. As expected, AuNPs containing more PMETAI exhibited greater activity against E. coli. Notably, using AuNPs with PMETAI:PMAG of either 3:1 or 5:1 at a low concentration of 12 nM, more than 98% inhibition in bacterial growth was observed after 3 h. PMAG was deemed necessary for efficacy as it improves the binding of nanoparticles to E. coli. In fact, the substantially lower MIC of the PMETAI-PMAG-coated AuNPs (i.e., 3 nM after an 18h incubation) compared to PMETAI alone suggested the synergistic functions of both PMETAI and PMAG, where selective binding to FimH on the E. coli cell surface due to PMAG possibly resulted in an increased localization of PMETAI on the bacterial cell wall. As FimH has a stronger affinity for mannose over other sugars, the composite nanoparticles could be recycled following detachment from bacterial cells through the addition of mannose. Retainment of efficacy after three consecutive uses was demonstrated. Further, the incorporation of PMAG to the hybrid nanoparticles was shown to mitigate the toxicity of PMETAI towards eukaryotic cells, as evidenced by the lack of toxic effects towards L929 fibroblast cells after a 3-day exposure to the nanoparticles (PMETAI:PMAG of 3:1) at concentrations equivalent to 2 to 4 × MIC.
Titanium(IV) oxide (TiO$_2$) is a unique antimicrobial compound due to its ability to disrupt bacterial cell walls and cause cell death by producing reactive oxygen species (ROS) in the presence of light [205, 206]. By combining the photocatalytic bactericidal effect of TiO$_2$ and the inherent antibacterial properties of amine-containing biocidal polymers, Kong et al. developed TiO$_2$-PTBAM-co-PEGDMA core-shell nanostructures with synergistic antibacterial properties in the absence and presence of light [197]. The formation of the polymer shell proceeded via surface-initiated photopolymerization using TiO$_2$ nanoparticles as an initiator. This resulted in rod-shaped nanostructures with an approximate length of 50 nm and a 2 nm thick polymer shell. When tested against *S. aureus*, pristine TiO$_2$ nanoparticles were only effective under ultraviolet (UV) irradiation, while bulk PTBAM-co-PEGDMA showed modest efficacy (i.e., 67% inhibition) with or without UV light. On the contrary, the TiO$_2$-polymer nanostructures resulted in 95.7% and 99.8% killing efficiency against *S. aureus* in the dark and after UV irradiation for 2 min, respectively. When the duration of UV irradiation was prolonged to 30 min, quantitative eradication of *E. coli* and *S. aureus* was observed for the core-shell nanostructures, while substantial viable cell populations were still detectable on the surfaces of the pristine TiO$_2$ nanoparticles. The enhanced ability of the hybrid composite nanoparticles to deactivate bacteria under both light and dark conditions was attributed to synergy between the TiO$_2$ nanoparticles and the biocidal PTBAM, as well as the high surface area-to-volume ratio of the nanoparticles compared to the bulk copolymer.

Recently, magnetic nanoparticles have proven useful in the biological field especially in applications such as cellular therapy, magnetic resonance imaging, tissue repair, targeted drug delivery, cell separation, and hyperthermia therapy [207, 208]. In particular, a special class of magnetic nanoparticles – termed superparamagnetic iron oxide nanoparticles (SPIONs) – has generated special interest as a result of their high biocompatibility compared to other metal or
metal oxide nanoparticles and the ability to ‘switch off’ their magnetic property after the removal of the external magnetic field [191]. Additionally, moderate activities against Gram-positive bacteria have been reported for SPIONs [209-211], which could potentially have synergistic antibacterial effects with other bactericidal compounds to form multi-modal antimicrobial agents. Despite the many unique properties of magnetic nanoparticles, the potential of magnetic nanoparticles as novel antimicrobials has not been extensively researched. The limited studies reported in this area typically focused on harnessing the magnetic property of the nanoparticles to afford recyclable antibacterial agents [212-214]. As magnetic nanoparticles in general still suffer from drawbacks related to surface oxidation, magnetically induced particle-particle aggregation and the lack of useful functional groups [215], researchers have looked into the surface modification of such nanoparticles with organic (polymer) coatings as a plausible solution to improve their applicability in the biomedical field [216].

A study reported by Kong et al. in 2010 on antibacterial γ-Fe₂O₃-polyrhodanine core-shell nanoparticles formed one of the earliest studies on the use of polymer-coated magnetic nanoparticles for antimicrobial applications [212]. The nanoparticles with average diameters of approximately 10 nm were synthesized in a one-step, organic solvent-free process, where released Fe³⁺ ions acted as oxidants for the polymerization of rhodanine monomers on the surface of the ferromagnetic core. Besides stabilizing the γ-Fe₂O₃ nanoparticles, the polyrhodanine shell was thought to provide antibacterial ability to the nanoparticles. In a preliminary antibacterial assessment, the hybrid nanoparticles were incubated with bacteria for 1 h at a concentration of 15 mg/mL, and were found to result in 90.2% and 99.9% reductions in the viable cell counts of E. coli and S. aureus, respectively. However, it is unclear as to whether the observed antibacterial activities were a result of synergistic killing by both γ-Fe₂O₃ and polyrhodanine, or purely due to the polymer. The recyclability of the nanoparticles by exposure to a magnetic field was also demonstrated, albeit with a slight reduction in efficacy where killing efficiency against S. aureus was reduced to 80% after five cycles.

Similarly, recyclable antibacterial nanoparticles in the form of polymer-coated ferromagnetic nanoparticles were developed by Matyjaszewski and co-workers [213]. Instead of the γ-Fe₂O₃ nanoparticles used in the previous study [212], a different core, Fe₃O₄, was coated with an antibacterial PQA. PDMAEMA brushes were polymerized from the Fe₃O₄ core using surface-initiated ATRP (SI-ATRP) to produce 50-150 nm particles, which were
then quaternized with ethyl bromide to afford a qPDMAEMA coating (Figure 1.24). While the bactericidal ability of the pristine Fe$_3$O$_4$ nanoparticles at 1 mg/mL was discounted, the qPDMAEMA-modified nanoparticles at the same concentration were able to eradicate *E. coli* completely after a 50-min incubation period. Further, the efficacy was retained quantitatively after eight cycles of repeated usage, where the nanoparticles were recovered with an external magnet after each use. The high potency exhibited by the nanoparticles against *E. coli* was attributed to the large nanoparticle surface area, the high density of biocidal quaternary ammonium groups, and the covalent attachment of polymer brushes on the surface.

**Figure 1.24.** Synthesis of qPDMAEMA-coated Fe$_3$O$_4$ nanoparticles via surface-initiated ATRP (SI-ATRP) [213]. Copyright 2011. Reproduced with permission from the American Chemical Society.

Sharma and co-workers reported the development of water-soluble SPIONs with a MnFe$_2$O$_4$ core and a multi-component shell consisting of carboxymethyl cellulose for water solubility, folic acid as a cancer cell-targeting moiety, and pH- and temperature-responsive poly(N-isopropylacrylamide)-co-poly(glutamic acid) (PNIPAm-co-PGA) [217]. While the ca. 37 nm core-shell nanoparticles, termed poly-SPIONs, were originally designed for the targeted delivery of anticancer hydrophobic drugs, they were also found to be antibacterial with a low MIC of 20 µg/mL against *E. coli*. The authors speculated that the small size of the poly-SPIONs facilitated internalization to bacterial cells and subsequent interaction with intracellular contents such as DNA, causing membrane damage and cell death. However, as the antibacterial mechanism of these nanoparticles remains elusive, further investigations, specifically to identify the antibacterial component in the nanoconstruct and the driving force for bacterial-nanoparticle interactions, are needed to yield more useful insights.

An interesting study was published by Jeong *et al.*, where recyclable nanoparticles capable of killing bacteria through near-infrared (NIR)-induced photothermolysis were developed [214]. Magnetic Fe$_3$O$_4$ nanoparticles were coated with a catechol-conjugated poly(vinylpyrrolidone) sulfobetaine (C-PVPS) inner layer and a poly(3,4-
ethylenedioxythiophene) (PEDOT) outer shell through layer-by-layer self-assembly (Figure 1.25). The incorporation of PVPS, which is a polyelectrolyte, was postulated to improve water solubility and native conduction efficiency. The role of PEDOT as a conducting polymer capable of absorbing NIR light (i.e., wavelengths of 750-950 nm) was substantiated as the Fe$_3$O$_4$@C-PVPS:PEDOT nanoparticles displayed a higher photothermal conversion efficiency than the pristine Fe$_3$O$_4$ nanoparticles. This translated to a higher killing efficacy shown by the functionalized nanoparticles, where quantitative killing of *E. coli* and *S. aureus* was achieved at a concentration of 1 mg/mL after 5 min of NIR irradiation. The authors postulated that the stronger photothermal activation achieved by the polymer-coated nanoparticles allowed the efficient release of thermal heat that was sufficient to induce irreversible bacterial destruction. This was in stark contrast to the mediocre antibacterial efficiency (i.e., 55-60% killing) of unmodified Fe$_3$O$_4$ nanoparticles under the same conditions. Further, surface modification had negligible effects on the superparamagnetic nature of Fe$_3$O$_4$ nanoparticles, and the Fe$_3$O$_4$@C-PVPS:PEDOT nanoparticles were shown to be recyclable with unaffected antibacterial activity for 3 cycles.

**Figure 1.25.** Schematic illustration of the synthesis of Fe$_3$O$_4$ nanoparticles coated with an inner PVPS layer and an outer PEDOT shell through layer-by-layer assembly [214]. Copyright 2015. Reproduced with permission from the American Chemical Society.

Extending from the concept of hyperthermia-induced bacterial cell death, Pu *et al.* investigated the possibility of combining magnetic-induced hyperthermia and treatment with membrane-disrupting polymers to achieve substantially enhanced antibacterial effects [218]. To achieve this goal, acid-functionalized cationic polycarbonates synthesized *via* organocatalyzed ROP of 5-methyl-5-(3-bromopropyl) oxycarbonyl-1,3-dioxan-2-one) (MTC-
O(CH$_2$)$_3$Br) monomers were quaternized and grafted onto the surface of superparamagnetic 10 nm MnFe$_2$O$_4$ nanoparticles through ligand exchange. The resulting nanoparticles had a $D_H$ of 17 nm with ca. 213 grafted polymer chains per nanoparticle. The free polymer chains (quaternized PMTC-O(CH$_2$)$_3$Br) were found to be bactericidal against *E. coli* and *S. aureus* only at a high concentration of 100 mg/mL, while the polymer-coated MnFe$_2$O$_4$ nanoparticles displayed a killing efficiency of 97-98% at 120 µg/mL, approximately 3 orders of magnitude lower than the effective concentration of the free polymer. Similar to earlier studies reported by other research groups [193, 213], the authors attributed the enhanced efficacy of the nanoparticles to the anchoring of the polymer brushes on the nanoparticle surface which led to an increase in local charge density. The postulation was substantiated by the lower antibacterial effectiveness demonstrated by nanoparticles with lower polymer grafting densities. Subsequently, the magnetic hyperthermia effect of the nanoparticles was investigated by introducing an alternating current magnetic field during incubation. Interestingly, the effect of hyperthermia was species-dependent. When tested against *E. coli*, near-quantitative (97%) and quantitative (100%) killing was achieved at 8 and 60 µg/mL, respectively. Although the bactericidal ability of the nanoparticles against *S. aureus* in the presence of a magnetic field was generally improved, the hyperthermia effect was less pronounced compared to that observed against *E. coli*, as complete eradication of *S. aureus* was only achieved at 120 µg/mL. Using MnFe$_2$O$_4$ nanoparticles with a PEG shell as a control, the authors demonstrated that the antibacterial properties of the quaternized PMTC-O(CH$_2$)$_3$Br)-coated MnFe$_2$O$_4$ nanoparticles were a result of the synergistic effects of magnetic hyperthermia induced by the metal oxide core and membrane damage caused by the cationic polymer shell.

1.3.3.4 **Other Core-Shell Polymeric Nanoparticles**

In 2006, Kenawy et al. reported the development of a class of insoluble polymeric biocides in the form of crosslinked core-shell nanoparticles for potential use in water treatment [219]. The nanoparticles were obtained from the copolymerization of vinylbenzyl chloride (VBC) with either 2-chloroethyl vinyl ether (CEVE) or methyl methacrylate (MMA) monomers in the presence of a cross-linker, divinylbenzene. This was followed by quaternization with trimethylamine, triphenylphosphine or tributylphosphine to confer antimicrobial properties to the nanostructures. A ‘cut plug’ method was used to evaluate the antimicrobial effects of the nanoparticles due to their water insolubility, where the copolymers were placed in wells composed of agar seeded with different microbial species.
Nanoparticles synthesized from VBC-CEVE copolymers and functionalized with triphenyl phosphonium salts were found to be the most effective against the range of bacteria and fungi species tested, producing inhibitory zones with diameters that ranged between 9 and 36 mm at a copolymer concentration of 5 mg. The antimicrobial ability of the nanoparticles was found to be species-specific. At a lower concentration of 2.5 mg/mL, the aforementioned VBC-CEVE nanoparticles were able to produce 70 and 90% killing against *C. albicans* and *S. aureus*; however, mediocre activity (≤70%) was demonstrated against *B. subtilis* and a fungal species *Aspergillus flavus*, even when the copolymer concentration was increased to 10 mg/mL.

In order to investigate the influence of shape on antibacterial core-shell nanoparticles, Chen and co-workers pioneered the fabrication of spherical, sheet-like and cylindrical nanostructures with polysiloxane cores decorated with densely grafted antimicrobial qPDMAEMA brushes [220]. Block copolymers of PDMAEMA and poly[3-(triethoxysily)propyl methacrylate] (PTEPM) were synthesized via RAFT polymerization and subjected to bulk microphase separation. This was followed by chemical cross-linking of the core, dispersal in acidic water to yield nano-objects with different morphologies depending on the PTEPM-to-PDMAEMA ratio, and subsequent quaternization with *n*-octyl bromide. While the sheet-like structures (54.1% PTEPM) were several µm in size, the nanocylinders (27.4% PTEPM) and nanospheres (14.5% PTEPM) possessed diameters of ca. 32 and 35 nm, respectively. Following a preliminary antibacterial assessment, the treatment of *E. coli* with any of the nanostructures, regardless of morphology, resulted in quantitative (>99.997%) cell death. Notably, the nanocylinders and nanospheres registered an MBC of 0.66 mg/mL, which is 10-fold lower than that of the qPDMAEMA homopolymer. This was in agreement with earlier studies, which theorized that an increased concentration and density of positive charges on the surface of a nanoparticle facilitated enhanced antimicrobial effects [113]. Despite the obvious differences in morphology and surface area across the sheet-like, spherical and cylindrical nano-objects, insignificant differences in terms of their activity against *E. coli* suggested that these factors were not dominant in the system investigated. The authors further postulated that other factors such as nanostructure size and the chain length of the grafted qPDMAEMA could be more crucial.

An interesting approach was utilized by Zhang *et al.* to prepare antibacterial, guanidine-based core-shell nanoparticles using a synthetic polymer template [221]. Using templated polymerization, acrylic acid and *N,N*-methylenebisacrylamide monomers were
copolymerized in the presence of PHMG in aqueous solution. Electrostatic interactions between poly(acrylic acid) (PAA) and PHMG led to the formation of insoluble interpolymer complexes which were then stabilized by PHMG oligomers at the periphery to remain water-dispersible, forming nanoparticles with an average $D_{H}$ of 190 nm. More than 2-log (>99%) reductions in E. coli and S. aureus cell counts were reported following treatment with 5 mg/mL of the PAA-PHMG nanoparticles, whereby activity was attributed to the PHMG shell. However, the effect of nanoparticle formation on the antibacterial performance of PHMG was inconclusive, as a comparison between the activities of the PAA-PHMG nanoparticles and PHMG homopolymers was not presented in the study.

Conductive polymers are a type of electroactive material that have generated increasing interest in the biomedical field due to their potential applications in tissue engineering, drug delivery, biosensing, and bioactuation, among others [222, 223]. To explore the potential of this unique class of polymers as novel antibacterial biomaterials, spherical nanoparticles (>200 nm) of poly(N-ethylaniline) (PNETA), a type of conductive polymer, were prepared by Chabukswar et al. via photocatalytic oxidative polymerization using tartaric acid as a dopant [224]. Interactions between the dopant and PNETA resulted in the organization of PNETA chains in a highly ordered manner to yield definite nanostructures. The PNETA nanoparticles were found to be the most effective against K. pneumoniae, with the most potent variants producing inhibitory zones between 28 and 29 mm and an MIC of 50 µg/mL. Moderate efficacies (i.e., inhibitory zones < 20 mm diameter) were exhibited against the other bacterial species tested, including E. coli, Salmonella typhi, B. subtilis, and S. aureus. The antibacterial properties of the nanoparticles were attributed to PNETA-bacteria electrostatic interactions, and possibly supplemented by the presence of the acidic dopants as well as the hydrophilic and hydrophobic groups in PNETA.

Emulsion polymerization has been recognized as one of the most common methods to synthesize polymer colloids such as core-shell polymeric nanoparticles [225, 226]. Despite being a relatively complex process governed by a multitude of factors, the fabrication of polymeric nanoparticles via emulsion polymerization constitutes an active research area, as the process is versatile and allows for facile control over particle size as well as the ability to prepare monodispersed and uniform particles [191].

In the field of antimicrobial research, the synthesis of poly(4-vinylpyridine) (P4VP)-based particles with bactericidal properties using an oil-in-water microemulsion
polymerization system was attempted by Ozay et al. in 2010 [227]. However, some of the particle variants synthesized were within the micron size range and in general, the polymers exhibited high MICs (10 mg/mL on average) against a range of Gram-positive and Gram-negative bacteria, even after quaternization or impregnation with metal nanoparticles. By combining the hydrophobicity of poly(acrylonitrile) (PAN) and the known antibacterial properties of poly(4-vinyl-N-alkylpyridinium, the same research group synthesized a series of PAN-co-P4VP-based core-shell particles by microemulsion polymerization [228]. The nanoparticles were post-modified by either converting the cyano groups on the PAN core to hydrophobic amidoxime groups, or quaternization of the P4VP shell to afford single or double positively charged materials. In general, the nanoparticles with diameters ranging from 270 to 520 nm demonstrated moderate efficacy against Gram-positive bacteria which included S. aureus and B. subtilis, but were non-active against Gram-negative bacteria. Taking into account their MICs and MBCs, the most effective variant registered MICs of 500 µg/mL against S. aureus and 250 µg/mL against B. subtilis, with equivalent MBCs of 1000 and 250 µg/mL, respectively.

Another research group reported the development of nanoparticles possessing a PMMA core and shells composed of chitosan, PEI, or a combination of chitosan and PEI through an emulsifier-free emulsion polymerization approach aided by an amine/tert-butyl peroxide initiating system [229]. The $D_h$ values of the nanoparticles were within the 140-162 nm size range. As a result of the amino groups present on chitosan and PEI, the nanoparticles were positively charged in an acidic environment. Further, for the PEI-containing nanoparticles, the buffering ability provided by PEI ensured stability and a constant positive charge of the nanoparticles over a wider pH range. When tested against bacteria, preferential activity against S. aureus over E. coli was displayed by all nanoparticle variants, with the highest activity shown by the nanoparticles containing a PEI only shell. The high antibacterial efficacy of PEI-coated nanoparticles was expected since PEI is a well-known membrane permeabilizer [230]. While the incorporation of chitosan resulted in reductions in activity to varying extents depending on the amount of chitosan added, the authors postulated that the presence of chitosan would impart biocompatibility to the otherwise toxic PEI nanoparticles; this, however, requires further investigations. The chitosan-PEI nanoparticles with 50 wt% PEI were demonstrated to be membrane-disruptive through SEM analysis, and showed comparable activity to the PEI only nanoparticles with MICs ranging from 1.7 to 3.2 mg/mL against S. aureus, and 2.8 to 4 mg/mL against E. coli.
Another area of interest in the development of core-shell nanoparticles with inherent antibacterial properties is the use of such nanoparticles as surface modifying agents to introduce antimicrobial functionalities to inert materials, such as cellulose fiber, textile, paper, and plastic [231]. To this end, Pan et al. developed a class of core-shell latexes based on the hydrophobic poly(butyl acrylate)-co-poly(ethylhexylacrylate) (PBA-co-PEHA) and antimicrobial macromonomers in the form of glycidyl methacrylate-modified PHMG (GPHMG) [231]. In the presence of a cationic emulsifier and the cross-linker EGDMA, emulsion polymerization was conducted in two stages to produce 104-155 nm nanoparticles, where the PBA-co-PEHA cross-linked core was first synthesized, followed by the formation of the hydrophilic GPHMG shell. The GPHMG shell was found to be essential for antibacterial efficacy, without which the nanoparticles became ineffective against *E. coli*. The (PBA-co-PEHA)$_{\text{core}}$GPHMG$_{\text{shell}}$ nanoparticles exhibited MICs ranging from 6.25 to 50 µg/mL against *E. coli*, where MIC was found to be inversely proportional to the GPHMG content. Notably, the best-performing nanoparticle with the highest (30.2 wt%) GPHMG content registered an MIC of 6.25 µg/mL, which was lower than that of the GPHMG homopolymer (MIC 8 µg/mL). It was hypothesized by the authors that nanoparticle-bacteria interactions were promoted by the small size and core-shell structure of the latex nanoparticles, resulting in the ability to inhibit bacterial growth at a relatively low dose.

Recognizing the drawbacks of conventional emulsion polymerization, especially those related to the use of large amounts of surfactant, Hazra et al. devised a facile route for the ‘green’ synthesis of 20-50 nm spherical PMMA$_{\text{core}}$biosurfactant$_{\text{shell}}$ nanoparticles by a modified microemulsion process [232]. The biosurfactants used – rhamnolipids, surfactin, and trehalose lipids – were biocompatible, biodegradable, non-toxic, and possessed low CMCs. When tested against *B. subtilis* and *P. aeruginosa*, the nanoparticles were generally more potent against *B. subtilis* with MICs of 0.1 to 0.32 mg/mL. Compared to pure biosurfactants and bulk PMMA, the higher antibacterial efficacies exhibited by the nanoparticles were attributed to larger surface areas, which provided more active sites for contact with bacterial cells. The PMMA$_{\text{core}}$surfactin$_{\text{shell}}$ (nPMMA$_{\text{SR}}$) nanoparticles displayed the highest activity, which was possibly due to the positively charged amine groups found only in surfactin but not in rhamnolipids and trehalose lipids. A mechanistic study performed on nPMMA$_{\text{SR}}$ using *B. subtilis* revealed that a combination of oxidative stress induction and cellular disintegration and fragmentation was likely to be involved in the antibacterial mechanism of the nanoparticles. In terms of their hemo-compatibility, nPMMA$_{\text{SR}}$ was found
to be the least hemolytic compared to bulk PMMA and the nanoparticles with rhamnolipid or trehalose lipid shells, although all three nanoparticle variants were relatively hemocompatible with less than 2% hemolysis at a high concentration of 1 mg/mL. The nanoparticles also demonstrated low toxicity to stimulated peripheral blood mononuclear cells, where at least 80% of the cells remained viable after a 5-day incubation with the nanoparticles at a concentration of 0.7 mg/mL.

Another approach utilizing polymerizable surfactants (or surfmers) for the synthesis of antibacterial core-shell nanoparticles to mitigate the surfactant-associated drawbacks of emulsion polymerization was reported by Li and co-workers [233]. Surfmers are amphiphilic compounds which possess the ability to act as surfactants and monomers [234], thereby enabling the incorporation of the surfactant to the synthesized nanostructure and enhancing nanoparticle stability while simplifying the nanoparticle purification process [235, 236]. A quaternary ammonium cationic surfmer of \( \text{N-(4-vinylbenzyl)-N, N-diethylamine hydrochloride (VEAH)} \) was introduced as a surfactant and a monomer for copolymerization with styrene to form polystyrene-co-PVEAH (PS-co-PVEAH) core-shell nanoparticles with average diameters between 96 and 204 nm. As expected, the nanoparticles with the highest PVEAH content (PS:PVEAH = 1:1) were found to be the most effective with MBCs of 15 and 20 mg/mL against \( \text{E. coli} \) and \( \text{S. aureus} \), respectively. It should be noted that the bacterial cell concentration used to determine the MBC value, \( 1 \times 10^7 \) colony forming units (CFU)/mL, was higher than that recommended by the Clinical and Laboratory Standards Institute (CLSI) [237] and typically used in other studies (i.e., a final concentration of \( 5 \times 10^5 \) CFU/mL for \( \text{E. coli} \) per well). By fabricating nanoparticles of varying sizes with an identical amount of quaternary ammonium moieties, the authors demonstrated that nanoparticles with smaller sizes displayed stronger antibacterial activities, which suggested that a large nanoparticle surface area is favorable for antibacterial efficiency similar to that previously reported [232].

1.4 Current Challenges and Limitations

Despite the vast amount of studies conducted on antibiotic alternatives and the therapeutic potential demonstrated by these novel compounds, the development of such antimicrobial agents is mostly stagnated at the discovery phase. Even in the field of AMPs, it was estimated that less than a few hundred peptides have been evaluated for clinical potential, which is significantly less than the number of candidates investigated in many antibiotic or
drug development programs [238]. This phenomenon could be attributed to several factors, which will be discussed below.

1.4.1 Multidrug-Resistant (MDR) Gram-Negative Bacterial Infections

MDR Gram-negative infections have been traditionally overshadowed by the focus on more well-known Gram-positive ‘superbugs’, such as MRSA and VRE. It was not until the emergence of the ‘ESKAPE’ pathogens – notorious for their role in the rising rates of nosocomial infections – in the past decade that Gram-negative infections became recognized as a more pressing concern and an emerging threat to human health [139, 140, 239]. The challenge is compounded by the paucity of new and effective drug candidates against such infections. The few drugs that remain effective against problematic MDR Gram-negative pathogens are mostly last-resort antimicrobials, such as polymyxins (polymyxins B and E, also known as colistin), which are potent but suffer from dose-limiting toxicity. Moreover, it was also found that a large number of antimicrobial polymers developed so far are selective against Gram-positive bacteria (refer to Section 1.3.3). This could be attributed to the fact that Gram-negative bacteria are harder to kill due to the presence of an outer membrane layer and additional resistance mechanisms not found in Gram-positive bacteria [139].

Additionally, the increasing number of studies that reported the development of resistance against AMPs or membrane-lytic antimicrobial agents in general further complicate the issue [39, 240]. As elucidated by Yeaman et al., constitutive or adaptive resistance mechanisms could be induced following AMP treatment, and include electrostatic shielding, the secretion of proteases and peptidases, as well as membrane structural modifications [241]. As most antimicrobial polymers currently investigated are designed to mimic the structure and function of AMPs, it is uncertain if resistance could be eventually developed against these polymers.

1.4.2 Toxicity

As highlighted in Section 1.3.1, the lack of cytocompatibility is a significant issue preventing the clinical development of AMPs. Even for polymyxins which have gained renewed interest for systemic use due to the increasing prevalence of MDR Gram-negative infections, their optimal use is limited by the high rates of polymyxin-induced nephrotoxicity [242, 243]. In studies related to antimicrobial polymers which are largely still at the basic research level, a greater emphasis is often placed on the antimicrobial performance of the polymers, rather than biocompatibility. Moreover, information derived from any in vitro or ex...
vivo assays conducted is limited and unable to accurately represent the toxicity profile of the investigated polymer in complex biological matrices and physiological settings in vivo [241].

1.4.3 Poor In Vivo Activity

A major issue hampering the development of some AMPs and antimicrobial polymers is their poor efficacy in vivo, in spite of their good performance when tested using in vitro antimicrobial susceptibility assays [63, 238]. This problem could be a result of the lack of standardization in the test medium used for in vitro studies. While full strength bacterial growth media are conventionally used in most studies, some publications were still reported based on antimicrobial data obtained using ‘modified’ media that were either diluted or had certain ionic species removed. The use of such ‘modified’ media could result in an overestimation of the compound’s actual efficacy. Furthermore, the culture broths used often do not reflect the biological conditions in vivo. It has been well-documented that inhibitory compounds such as salts and proteins found under physiological conditions, as well as other factors such as temperature and pH could have substantial effects on the activity of cationic antimicrobial agents [63]. On the other hand, it was also demonstrated that many pathogenic bacteria are more virulent in minimal media than in rich growth conditions [244, 245]. Hence, it is vital to evaluate antimicrobial efficacy in a range of settings to better elucidate the complex interactions between an antimicrobial agent and its environment.

1.4.4 Limited Scope of Study

It is noteworthy that most studies on the development of antimicrobial polymers relied on end-point measurements (e.g., MIC and HC50) to assess the efficacy and cytocompatibility of the polymers. While the approach is efficient and suitable as an initial screening analysis, the lack of follow-up studies, such as to elucidate the impact of polymer structure on activity, biocompatibility, bacterial resistance acquisition, and antimicrobial mechanism, restricts the proper understanding of the biological properties of such compounds. Further, most mechanistic studies are confined to the bacterial membrane morphology, as it is commonly assumed that antimicrobial polymers behave similarly to membrane-active AMPs. However, numerous studies have shown that AMPs possess much more complex modes of action beyond membrane disruption [32]. Different AMPs have been shown to act on different bacterial target sites with different mechanisms of action. Collectively, the limited scope of study on antimicrobial (peptide) polymers could hinder their potential for clinical translation.
1.5 Research Objectives

Considering the limitations and challenges facing the development of new drugs to combat antimicrobial resistance, the central research theme of this thesis was to develop novel antimicrobial agents that (i) possess high efficacy against MDR (Gram-negative) pathogenic bacteria, (ii) demonstrate low or minimal susceptibility to resistance development, and (iii) are highly biocompatible with mammalian cells. Building upon our expertise in peptide-based polymeric materials engineering, a new class of nano-sized, peptide-based macromolecular antimicrobials in the form of star polymers, termed ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs), was developed. The research was first aimed at evaluating the in vitro efficacy of SNAPPs against a range of clinically-relevant bacteria and their toxicity towards mammalian cells. The polymers were then subjected to in vivo performance tests using a mouse model, resistance studies, and detailed mechanistic investigations. The research was followed up by a detailed investigation on the structure-activity relationship of SNAPPs by focusing on the effects of polymer structure (star arm copolymer structure as well as overall macromolecular architecture) on antibacterial efficacy and biocompatibility. Subsequently, a bio-nano interaction study was conducted to evaluate the effects of different medium environments (including in vivo-mimicking settings) on the antibacterial activity of SNAPPs and their mechanism of action. Lastly, the synergistic interactions between SNAPPs and common antibiotics were explored with the aim of developing a combination treatment against MDR bacteria that is effective and safe.
Figure 1.26. Research theme of the thesis, which is the development of novel antimicrobial agents in the form of star peptide polymers, referred to as ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs).

1.6 Thesis Outline

In Chapter 2, the synthesis of SNAPPs, in the form of 16- and 32-arm star peptide polymers, through the NCA-ROP of lysine and valine amino acid monomers is presented (Figure 1.27). SNAPPs were evaluated for their in vitro antimicrobial properties against a range of Gram-negative and Gram-positive bacteria, including MDR bacterial species, and their performance was compared with those of their linear ‘one star arm’ equivalent and AMPs. Resistance studies were conducted using wild-type and MDR bacteria to evaluate if resistance against SNAPPs could be generated easily. The biocompatibility of SNAPPs was assessed by determining their hemocompatibility and toxicity towards mammalian cells. Using a mouse peritonitis model, the efficacy of SNAPPs to combat wild-type and MDR Acinetobacter baumannii infections in vivo was investigated. Subsequently, mechanistic studies, in the form of fluorescence and electron microscopy, competitive inhibition assays, flow cytometry, membrane disruption measurements, and gene expression studies, were performed to study the mechanism of action of SNAPPs.
Figure 1.27. Chapter 2: Chemical structure of antimicrobial star-shaped peptide polymers, referred to as ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs).

Chapter 3 presents a study designed to address the research questions raised from the findings reported in Chapter 2. These include the effects of (1) the star arm (co)polymer structure and (2) the star architecture on the antimicrobial activity and biocompatibility of SNAPPs (Figure 1.28). A library of star and linear peptide polymers was established, which composed of 16- and 32-arm stars with block, random, or homopeptide arms as well as their linear analogues. Advanced flow cytometry was combined with conventional antimicrobial susceptibility assays to provide a comprehensive evaluation of the antimicrobial activity and membrane disrupting abilities of the polymers. The biocompatibility of the polymers within the library was compared in terms of their hemolytic activity and cytotoxicity. To elucidate the possible effect of peptide secondary structure, circular dichroism was performed in a membrane-mimicking environment.

Figure 1.28. Chapter 3: Understanding the effects of polymer structure on antimicrobial activity and biocompatibility.
In Chapter 4, a bio-nano interaction study between SNAPPs and biological molecules present in vivo is described (Figure 1.29). The antibacterial efficacy of SNAPPs against a range of clinically-relevant Gram-negative bacteria was assessed in media containing physiologically relevant concentrations of salts and proteins. This was supplemented by outer and inner membrane permeability assays using fluorometric techniques and flow cytometry. Based on the research findings, the effects of (bio)molecules, namely divalent cations and proteins, on the antibacterial mechanism of SNAPPs were deduced, and a strategy to overcome the inhibitory effects of divalent cations was demonstrated.

**Figure 1.29.** Chapter 4: Investigating the bio-nano interactions of star peptide polymers (SNAPPs) in in vivo-mimicking environments.

Chapter 5 explores the synergistic interactions between SNAPPs and different classes of antibiotics against Gram-negative bacteria and the ability of SNAPPs to restore the efficacy of antibiotics against drug-resistant species through co-administration. A screening analysis for synergy was first conducted by evaluating bacteria viability after treatment with sub-lethal concentrations of SNAPPs and antibiotics, either individually or in combination. Based on the results, the degree of synergy for each SNAPP-antibiotic combination was computed through the Bliss and Highest Single Agent models. For selected combinations where synergy was demonstrated, the effects of antibiotic and SNAPP concentrations on the degree of synergy were further investigated. Lastly, the ability of SNAPP-antibiotic synergy in mitigating toxicity towards mammalian cells is presented.
In **Chapter 6**, concluding remarks for each chapter are discussed, along with the proposed future directions for the overall body of work outlined in this thesis. A summary of characterization methods and detailed experimental procedures are provided in **Chapter 7**.

### 1.7 References


CHAPTER 2

Combating Multidrug-Resistant Gram-Negative Bacteria with Structurally Nanoengineered Antimicrobial Peptide Polymers

2.1 Chapter Perspective

In this chapter, a new class of antimicrobial agents, termed ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs) was developed and found to exhibit sub-µM activity against all Gram-negative bacteria tested, including ESKAPE and colistin-resistant and MDR (CMDR) pathogens, while demonstrating low toxicity. SNAPPs were highly effective in combating CMDR Acinetobacter baumannii infections in vivo, the first example of a synthetic antimicrobial polymer having CMDR Gram-negative pathogen efficacy. Further, no resistance acquisition by A. baumannii (including the CMDR strain) to SNAPPs was observed. Comprehensive analyses using a range of microscopy and (bio)assay techniques revealed that the antimicrobial activity of SNAPPs proceeded via a multi-modal mechanism of bacterial cell death by outer membrane destabilization, unregulated ion movement across the cytoplasmic membrane and induction of the apoptotic-like death pathway, possibly accounting for why resistance to SNAPPs was not detected in CMDR bacteria. Overall, this chapter demonstrated that SNAPPs are promising as low-cost and effective antimicrobial agents, and may represent a weapon in combating the growing threat of MDR Gram-negative bacteria.
2.2 Introduction

A group of pathogens responsible for the majority of hospital-acquired infections – commonly referred to as the “ESKAPE” pathogens (i.e., Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter) – have been named as one of the biggest threats to health as a result of their multidrug resistance [1-3]. Although the Gram-positive bacteria in the ESKAPE group, including the methicillin-resistant S. aureus, have rightly drawn attention over the past decade, infections caused by the Gram-negative microbes have been recently recognized as a more critical healthcare issue [4,5]. Despite the fact that many Gram-negative bacteria have acquired antibiotic resistance, the pipeline for the development of new antimicrobials that target Gram-negative bacteria remains empty [6]. The dearth of drug candidates against Gram-negative bacteria is attributed to the fact that they might be harder to kill compared to Gram-positive bacteria, largely due to the presence of an outer membrane (OM) that serves as a highly impermeable barrier, as well as additional defense mechanisms that might be absent in Gram-positive bacteria [4,7].

Antimicrobial peptides (AMPs) have been widely regarded as a promising solution to combat MDR bacteria [8]. Unlike conventional antibiotics that act on specific intracellular targets [9], AMPs interact with microbial membranes through electrostatic interactions and physically damage the bacterial morphology [10]. The nature of this antimicrobial mechanism renders bacteria less likely to develop resistance against AMPs. However, AMPs have had limited success in clinical settings (with the exception of a few promising candidates being evaluated as topical agents, such as omiganan [11]), primarily due to their high toxicity towards mammalian cells [12]. Recently, ring-opening polymerization (ROP) of α-amino acid N-carboxyanhydrides (NCAs) has been shown to be a versatile method for the synthesis of peptide polymers with antimicrobial properties [13, 14], even though NCA-ROP cannot rival the precise peptide sequences obtained via solid-phase peptide synthesis. Nevertheless, advances in NCA-ROP have provided a facile route for the synthesis of well-defined peptide polymers with complex macromolecular architectures, such as star polymer nanoparticles [15, 16]. Several research groups have recently reported studies that demonstrated the potential of these star-shaped peptide polymer nanoparticles in nanomedicine [17], particularly in the fields of gene therapy [18,19] and targeted drug delivery [20].
This chapter presents the discovery of star-shaped peptide polymer nanoparticles consisting of lysine and valine residues, synthesized via NCA-ROP, as a new class of antimicrobial agents. These star nanoparticles were termed ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs). Unlike existing self-assembled antimicrobial macromolecules that will dissociate to unimers below their critical micelle concentration [21,22], SNAPPs are stable unimolecular architectures up to infinite dilution. SNAPPs were found to exhibit superior antibacterial activity against a range of clinically-important Gram-negative bacteria, possess high therapeutic indices, and display selectivity towards pathogens over mammalian cells. Crucially, SNAPPs were able to combat CMDR A. baumannii infection in mice. SNAPPs were shown to possess a multi-modal antimicrobial mechanism involving disruption of the integrity of the OM, cytoplasmic membrane (CM) disruption, unregulated ion efflux/influx, and induction of apoptotic-like death (ALD), which potentially accounts for the superior performance of SNAPPs and differs from that typically reported for most AMPs.

2.3 Results and Discussion

2.3.1 Synthesis and Characterization of SNAPPs

To demonstrate the potential of this new class of antimicrobial nanomaterial, SNAPPs in the form of 16- and 32-arm star peptide polymer nanoparticles S16 and S32, respectively, were synthesized via NCA-ROP (number-average molecular weight, \(M_n = 43.8 \text{ kDa (S16)}, 74.8 \text{ kDa (S32)}\); hydrodynamic diameter, \(D_H = 7.7 \text{ nm (S16)}, 13.5 \text{ nm (S32)}\); see Figure 2.1; Appendix I, ‘Synthesis and Characterization of SNAPPs’ section, Supplementary Figures 2.1-2.8, Supplementary Table 2.1), in a similar fashion to that previously reported [18]. The number of star arms was fixed at 16 or 32 based on the availability of the dendritic precursors and to maintain consistency with the previous work [18]. Inspired by naturally-occurring AMPs where the mode of action has been partly attributed to a positively charged amphipathic structure [8] and after taking into account various synthetic considerations, lysine and valine were selected as cationic and hydrophobic amino acids, respectively. Their monomeric NCA derivatives were randomly polymerized from a poly(amido amine) (PAMAM) dendritic core to form the star arms with a theoretical lysine-to-valine ratio of 2:1, which was selected to promote water solubility, and an average degree of polymerization (DP) of 30 residues per star arm at complete monomer conversion.
Figure 2.1. Synthesis of SNAPPs. Synthesis of SNAPPs via ring-opening polymerization (ROP) of lysine and valine N-carboxyanhydrides (NCAs) was initiated from the terminal amines of poly(amido amine) (PAMAM) dendrimers. Second (G2) and third (G3) generation PAMAM dendrimers (see Appendix I, Supplementary Figure 2.2 for structure of the former) with 16 and 32 peripheral primary amines were used to prepare 16- and 32-arm SNAPPs, respectively. Note that the number of initiating points on the figure does not reflect the actual number which is 16 or 32. The number of repeat units for lysine and valine are $a$ and $b$, respectively. The lysine-to-valine ratios (i.e., $a:b$) are provided in Appendix I, Supplementary Table 2.1.

2.3.2 In Vitro Antimicrobial Properties of SNAPPs.

The antibacterial efficacy of SNAPPs was evaluated by determining their minimum bactericidal concentrations (MBCs) against a range of Gram-positive (*Streptococcus mutans* and *S. aureus*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *A. baumannii*) bacteria. The MBC is defined as the minimum drug concentration that causes quantitative cell death (see Chapter 7.4 for experimental methods; Appendix I, Supplementary Figure 2.9 and Supplementary Table 2.2 for further clarification). The antimicrobial susceptibility assays were initially conducted in Mueller-Hinton broth (MHB), a nutrient-rich bacterial growth medium. Initial studies which focused on the two Gram-positive bacteria (*S. aureus* and *S. mutans*) and two Gram negative bacteria (*E. coli* and *P. aeruginosa*) showed that $S_{16}$ and $S_{32}$ had preferential activity towards the Gram-negative species (MBC<1.4 μM) (Table 2.1) over the Gram-positive strains (MBC>1.8 μM).
It is worthwhile noting that homolysine star-shaped peptide polymer nanoparticles, i.e. those that do not contain valine residues, exhibited a higher MBC value (>3-fold increase in MBC against *E. coli*), thus demonstrating the need for an amphipathic structure to effect antimicrobial activity.

**Table 2.1.** Antimicrobial Activity of SNAPPs and Other Peptides against a Range of Gram-Negative Pathogens.

<table>
<thead>
<tr>
<th>Antimicrobial type</th>
<th>Code/Name</th>
<th>Media</th>
<th>MBC(^a), µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td><strong>SNAPP</strong></td>
<td>S16</td>
<td>MHB</td>
<td>0.72 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>MHB</td>
<td>0.72 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>S32</td>
<td>MHB</td>
<td>0.72 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>MHB</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td><strong>AMP</strong></td>
<td>Ovispirin(^c)</td>
<td>MHB</td>
<td>8.39 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>Magainin II(^c)</td>
<td>MHB</td>
<td>47.85 ± 6.08</td>
</tr>
<tr>
<td></td>
<td>Melittin(^c)</td>
<td>MHB</td>
<td>33.71 ± 5.18</td>
</tr>
</tbody>
</table>

\(^a\)MBC is defined as the minimum drug concentration that causes quantitative bacterial cell death (refer to Chapter 7.4, and Appendix I, Supplementary Figure 2.9 and Supplementary Table 2.2 for further clarification). All data are expressed as mean and standard deviations of four replicates (\(n = 4\)) completed in two independent experiments. \(^b\)The MBC values obtained were identical across all replicates. \(^c\)The amino acid sequences of ovispirin, magainin II and melittin are KNLRRIIRKIIHIKKG-COOH, GIGKFLHSAKFGKAFGVEIMNS-COH2, and GIGAVLKVLTGPLALISWIKRRQQ-COOH, respectively. Ovispirin, magainin II and melittin were synthesized using standard solid-phase peptide synthesis protocols for Fmoc (9-fluorenlymethoxy carbonyl) chemistry (refer to Chapter 7.5 for details) and their antimicrobial activities were evaluated as per SNAPPs.

In further studies using Gram-negative bacteria, **S16** and **S32** were also found to be effective against *K. pneumoniae* and *A. baumannii*, with **S16** registering MBC values of 1.54 and 0.85 µM, respectively, and **S32** showing similar MBC values of 0.83 and 0.79 µM. Further, both **S16** and **S32** were found to be equally effective against CMDR clinical isolates of *P. aeruginosa* (FADDI-PA067) and *A. baumannii* (FADDI-AB156) (Appendix I, Supplementary Tables 2.4-2.5) as to drug-sensitive strains, yielding MBC values that range from 0.85 to 1.61 µM (Table 2.1). The MBC values of SNAPPs against all bacteria tested were found to be within a similar order of magnitude, which implied that the antimicrobial efficacies of **S16** and **S32** might not be species-specific for Gram-negative bacteria. This was surprising as *P. aeruginosa* and *K. pneumoniae* possess low antibiotic susceptibility as a
result of their thick extracellular capsules, which contribute to reduced cell permeability [23] and is a resistance mechanism against antibiotics and AMPs [24, 25]. Noteworthy, the probability of the PAMAM dendrimer cores contributing to the antimicrobial efficacies of S16 and S32 in this study was discounted, as the PAMAM cores were found to be non-active against the range of bacterial species tested above (Appendix I, Supplementary Table 2.6).

Further, an antimicrobial resistance study was conducted to evaluate if resistance against SNAPPs could be generated easily. Following serial passaging of bacterial cells in the presence of sub-MBC levels of S16, de novo resistant mutants of wild type and CMDR A. baumannii to S16 were not obtained even after 600 generations of growth (over a period of 24 days) (Appendix I, Supplementary Figure 2.10). The MBC values of S16 against these bacterial strains remained relatively constant throughout the experiment, despite the fact that the CMDR strain is genetically capable of mutation and rapid resistance acquisition. This result suggested that resistance to SNAPPs is not acquired easily.

As a comparator, the linear analog L was synthesized to represent one arm of S16 and S32 (Appendix I, Supplementary Figures 2.11-2.13, Supplementary Table 2.7). Interestingly, compared to SNAPPs, the linear analog was poorly active against E. coli and S. aureus, with MBCs that were at least 40-fold higher than those of SNAPPs (Appendix I, Supplementary Table 2.8). It was hypothesized that the star architecture has a significant effect on enhancing the antimicrobial action of random co-peptide polymers against bacteria. Similar to that observed by Yang and co-workers, it was further theorized that the improvement in activity is a result of the increased local concentration of charges provided by the nanostructure, thereby leading to greater ionic interactions with the bacterial membranes [22]. Additionally, bacterial membrane-induced peptide aggregation has been postulated to be a key factor of AMP efficacy, as it enables AMPs to achieve the high threshold concentrations needed for membrane disruption [26]. Extending from this concept, it was hypothesized that the star architecture affords a high local concentration of peptide mass even in solution before contacting bacterial cells, which may contribute to the enhanced efficacy of SNAPPs.

The antimicrobial activities of S16 and S32 were compared with several peptide-based antimicrobial agents known to be effective against Gram-negative pathogens, including magainin II, ovispirin and melittin (Table 2.1). The antimicrobial efficacies of these AMPs against certain Gram-negative pathogens have been well-documented; however, they (as with most AMPs) tend to demonstrate selective activity towards certain bacterial strains, even
within the Gram-negative family (Table 2.1). This is in direct contrast to SNAPPs which displayed effective equipotent activity against all of the Gram-negative species tested. Furthermore, SNAPPs were orders of magnitude more effective than these AMPs (Table 2.1).

To explore the antimicrobial efficacy of SNAPPs in different media and to evaluate their toxicity against mammalian cells, the assays were repeated using minimal essential medium (MEM), which is a defined medium for mammalian cells. Both S16 and S32 exhibited at least four times lower MBC values (<0.19 µM) against all bacterial species tested in MEM compared with MHB. In MHB, SNAPPs were found to aggregate to form particles with average $D_H$ values of ~ 224.2 nm or greater, which is significantly larger in size compared to SNAPPs in MEM ($D_H = 7.7$ and 13.5 nm for S16 and S32, respectively) (Appendix I, Supplementary Figures 2.6-2.7). Cryo-TEM images of S16 in MHB further confirmed that the star formed large aggregates (~ 200-500 nm in diameter) (Appendix I, Supplementary Figure 2.14). The formation of aggregates is consistent with previous studies that have reported that nutrient/ion-rich media often contain anionic peptide and protein fragments, which might bind non-specifically to cationic antimicrobials [27], thereby causing aggregation and the formation of larger-sized particles in MHB as observed herein. This postulation was validated as a similar phenomenon was observed where aggregates of S16, predominantly sized at 125 nm, were formed in 1% fetal bovine serum, another protein-rich medium (Appendix I, Supplementary Figure 2.15). The aggregation of SNAPPs with the medium contents could possibly shield the active components of SNAPPs, thereby reducing their potency. Nevertheless, both S16 and S32 still possessed high efficacies in MHB, especially compared to the lead AMPs.

2.3.3 Biocompatibility of SNAPPs

As a test of biocompatibility, the hemolytic activities of SNAPPs were investigated by incubating them with red blood cells at different nanoparticle concentrations. Both S16 and S32 (as well as the control homolysine star) had negligible hemolytic activity (>45 µM; Appendix I, Supplementary Table 2.9). Even at a very high concentration of >100 × MBC, the extent of hemolysis was well below 30% (Appendix I, Supplementary Figure 2.16). Subsequently, the viability of two types of mammalian cells, human embryonic kidney (HEK293T) cells and rat hepatoma (H4IIIE) cells, in response to SNAPPs was investigated. The therapeutic indices (TI) of SNAPPs ranged from 52 to 171 (Appendix I, Supplementary
Table 2.10), generally higher than the TI of colistin (also known as polymyxin E) [28] which is now being used as the last therapeutic option for MDR Gram-negative pathogens [29].

2.3.4 *In Vivo* Efficacy of SNAPPs

The effectiveness of S16 *in vivo* was evaluated in a mouse peritonitis model, where the intraperitoneal (i.p.) dose of *A. baumannii* (2 x 10^8 cells in MEM) resulted in the establishment of wide-spread bacterial infection by 24 h (Figure 2.2a). At 0.5, 4 and 8 h post-infection, mice were treated with either MEM (control), the antibiotic imipenem (40 mg/kg), or S16 (8.3 mg/kg). Similar to the imipenem-treated mice, treatment with S16 resulted in >5-log reduction in bacterial cell counts in the peritoneal cavity (Figure 2.2b), quantitative (>99%) eradication of bacterial cells in blood (Appendix I, Supplementary Figure 2.17a), and >3-log CFU reduction in the spleen (Appendix I, Supplementary Figure 2.17b). Additionally, all mice treated with either imipenem or S16 survived with no signs of animal distress, whereas only 20% of the control/mock-treated mice survived after 24 h. A number of studies have found that antimicrobial agents enhance host cell innate immunity to bacteria *in vivo* [30]; in this study S16-treated group enhanced neutrophil infiltrate in the peritoneal cavity, while the imipenem-treated group did not show any significant difference from the mock-treated group (Figure 2.2d).
Figure 2.2. In vivo efficacy of SNAPP S16 in a mouse peritonitis model. 

**a**, Schematic of the experimental protocol for the mouse peritonitis model. **b-c**, Colony forming units (CFU) of *A. baumannii* (ATCC 19606) (**b**) and CMDR *A. baumannii* (FADDI-AB156) (**c**) found in the peritoneal wash of infected mice 24 h after mock (MEM) treatment or treatment with imipenem (40 mg/kg) or S16 (8.3 mg/kg). **d-e**, Numbers of peritoneal neutrophils in the mild peritonitis model with mice 24 h after infection with *A. baumannii* (ATCC 19606) (**d**) and CMDR *A. baumannii* (FADDI-AB156) (**e**) and either mock, imipenem (40 mg/kg) or S16 (8.3 mg/kg) treatments. All data are expressed as mean ± standard deviation as indicated by the error bars, based on values obtained from five biological replicates (*n* = 5). *P* < 0.01, **P** < 0.001, Student’s *t* test, significant difference from the mock (MEM) control group (**b**, **c**) and the imipenem-treated group (**d**, **e**).
As S16 was effective in vitro against CMDR bacteria, the peritonitis model was extended to include the CMDR A. baumannii. Mice treated with S16 had significantly less bacteria in the peritoneal cavity (Figure 2.2c), blood (Appendix I, Supplementary Figure 2.18a) and spleen (Appendix I, Supplementary Figure 2.18b), and higher numbers of neutrophils in the peritoneal cavity (Figure 2.2e) compared with the imipenem- and mock-treated groups. Imipenem treatment had no effect on reducing bacteria levels in all tissues examined and this was comparable to the mock-treated group (Figure 2.2c; Appendix I, Supplementary Figure 2.18). While all mice treated with S16 survived with no signs of animal distress, only 50% of the mock- or imipenem-treated mice survived the 24 h infection. The mechanism by which host defense peptides (HDPs) effect bacterial clearance in vivo is by neutrophil recruitment; however, unlike SNAPPs, HDPs often have poor direct antimicrobial activity [31]. S16 demonstrated both direct (bacterial) and indirect (via neutrophil recruitment) antimicrobial activities in vivo. While the CMDR A. baumannii isolate used herein has been found to acquire resistance against the last-resort drug, colistin [32], these results demonstrated that S16 is capable of treating CMDR A. baumannii and potentially other CMDR Gram-negative infections in vivo. To the best of our knowledge, this is the first report of a synthetic antimicrobial polymer having in vivo efficacy against a CMDR Gram-negative bacterial infection.

2.3.5 Preliminary Mechanistic Studies

To directly observe the interactions between an antimicrobial agent and bacterial cells, super-resolution fluorescence imaging was conducted using 3D-Structured Illumination Microscopy (3D-SIM). Sample images of untreated E. coli are provided in Figure 2.3a and Supplementary Figure 2.20a (Appendix I). Figure 2.3 shows the 3D-SIM images of E. coli (labelled red with lipid membrane FM4-64FX dye) incubated with the AF488-labelled S16 (Appendix I, Supplementary Figure 2.19, Supplementary Table 2.11) in MHB at a dose approximately equivalent to 0.5 ×, 1 × and 2 × the MBC of the fluorescently tagged SNAPP (MBC_tagged). While the action of antimicrobials on bacteria has been imaged using a range of microscopy techniques [33,34], this is one of the first instances whereby a clear visualization of the interaction between an antimicrobial agent and bacterial cells at the super-resolution level has been demonstrated. S16 (labelled green) was found to associate at certain sites on and in the bacteria depending on the SNAPP concentration (Figure 2.3b-h; Appendix I, Supplementary Figures 2.20-2.21). This is different to the membrane interactions of proline-rich AMPs, where the peptides localized uniformly around the E. coli membrane as
previously reported [35]. At 0.5 × MBC\textsubscript{tagged}, SNAPPs associated with the surface of the bacteria (Figure 2.3b); however, at 1 × MBC\textsubscript{tagged}, a high density of bacterial cells with either membrane associated or internalized star peptide polymers was observed (Figure 2.3c-e). On the other hand, in the previous study on membrane-lytic proline-rich AMPs, complete internalization and uniform localization of peptides throughout the cytosol of the bacterial cells were observed [35]. This difference was attributed to the larger sizes of SNAPPs, which possibly inhibited the quantitative internalization of all membrane-bound SNAPP macromolecules even when the membranes were disrupted. At 2 × MBC\textsubscript{tagged}, there was clearly more membrane-associated or internalized SNAPPs per bacterial cell (Figure 2.3f-h; Appendix I, Supplementary Figure 2.20b-e). Orthogonal projections showed that the membrane localized SNAPPs appeared to aggregate and span the cell envelope (Appendix I, Supplementary Figure 2.21). Further, bacteria with internalized SNAPPs were observed to have distinct cell envelope perforations (Figure 2.3h; Appendix I, Supplementary Figures 2.20d-e and 2.21b). When the experiments were conducted in MEM at 2 × MBC\textsubscript{tagged}, the results were similar to that observed in MHB at supra-MBC dosage, albeit with a larger extent of SNAPP internalization into the cells (Appendix I, Supplementary Figure 2.22).

Figure 2.3. OMX 3D-SIM images of \textit{E. coli} before and after treatment with AF488-tagged SNAPP S16 in Mueller-Hinton broth (MHB). a-h, Z-projection images of \textit{E. coli} before (a) and after incubation with AF488-S16 at 0.5 × MBC\textsubscript{tagged} (b), 1 × MBC\textsubscript{tagged} (c-e), and 2 × MBC\textsubscript{tagged} (f-h). Scale bars, 1 µm. The \textit{E. coli} cell membrane was stained with FM4-
64FX (red) and S16 with AF488 (green) in all images. Note that the MBC used refers to the MBC of the fluorescently tagged SNAPP (Appendix I, Supplementary Table 2.11). All images are representative of three independent experiments.

Based upon the fluorescence imaging studies conducted, it was hypothesized that SNAPPs initially localize on the bacterial OM as a result of electrostatic interactions. This could cause areas of destabilization/fragmentation leading to SNAPPs possibly translocating to and disrupting the CM, thus leading to cell death [36]. To investigate this hypothesis, a competitive inhibition assay with LPS was conducted. The co-incubation of LPS (from E. coli) with S16 was found to inhibit the ability of S16 to disrupt the membrane of E. coli cells in a dose-dependent manner (Appendix I, Supplementary Figures 2.23-2.24). This suggested that SNAPPs bind to LPS on the OM and could explain the selective antimicrobial activity of SNAPPs towards Gram-negative bacteria. However, as SNAPPs were still moderately active against the Gram-positive species tested, it was postulated that the LPS-SNAPP interactions are preferred, due to the strong electrostatic interactions, but not highly specific to the extent where antimicrobial activity would be lost in the absence of LPS. Using flow cytometry, it was demonstrated that the association of the AF488-tagged S16 with E. coli was linearly correlated with bacterial membrane disruption (Appendix I, Supplementary Figures 2.25-2.26). Furthermore, a time-course study showed that the action of S16 was rapid as >90% of an E. coli cell population had disrupted membranes within 30 min which correlated with complete population death (Appendix I, Supplementary Figure 2.27). This fast kinetics of killing is similar to that observed for AMPs [37], which is most likely a distinctive feature of membrane-disruptive antimicrobials.

To investigate if SNAPP-induced membrane disruption is a result of pore formation like some AMPs, a dye release assay was conducted using dextran-loaded large unilamellar vesicles (LUVs) as a mimic for Gram-negative CM [38]. The results suggested that SNAPPs did not cause membrane disruption via pore formation (Appendix I, Supplementary Figure 2.28a). Another mechanism of membrane disruption is through unregulated ion movement, which was investigated using the LUV chloride ion (Cl\(^-\)) transport assay [39]. Cl\(^-\) ion efflux was found to increase with increasing concentrations of S16 (Appendix I, Supplementary Figure 2.28b). Additionally, membrane potential measurements were performed on bacterial cells to determine the ability of SNAPPs to alter membrane potential. It was observed that the treatment of E. coli cells with S16 induced mixed hyperpolarized and depolarized bacterial cell populations, with a shift towards a more depolarized population as concentration
increased (Appendix I, Supplementary Figures 2.29-2.30). Taken together, these studies suggested that the interaction of SNAPPs with the CM may result in membrane perturbations that led to unregulated ion movement and membrane potential dissipation.

A number of recent studies have shown that bacteria, like eukaryotic cells, have mechanisms of programmed cell death (PCD) that could be triggered under stressful conditions, such as membrane disruption [40,41]. Two major PCD pathways have been described in bacteria: (i) the ALD pathway mediated by recA and lexA genes, and (ii) the mazEF pathway [40,41]. Based on gene expression studies, S16 at 1 × MBC induced a 10- and 7-fold increase in recA and lexA, respectively, but no change in mazEF levels (Appendix I, Supplementary Figure 2.31). These results, when observed under cell death conditions, suggested that S16 induced ALD responses in E. coli. Further, the production of reactive oxygen species (ROS) following S16 treatment (Appendix I, Supplementary Figure 2.32), which has been reported to be a characteristic of ALD [42], was observed. The induction of ALD in bacteria has been reported in previous studies to lead to cell lysis [42]. It was also found that when ALD was inhibited by pre-treating E. coli with a translation inhibitor (doxycycline), the antimicrobial activity of S16 remained comparable to that when ALD was not inhibited (Appendix I, Supplementary Figure 2.33). This suggested that the induction of the ALD pathway is not a prerequisite for SNAPP activity, but likely to be either an event that coincides with early membrane disruption or a supplementary (but not essential) bactericidal mechanism. At 5 × MBC, S16 induced significantly less recA and lexA mRNA than that at 1 × MBC concentration (Appendix I, Supplementary Figure 2.31). It was speculated that at supra-MBC dosage other killing mechanisms would dominate, thus leading to insufficient time for the expression of ALD pathway components. This agreed with the aforementioned postulation that there might be multiple mechanisms involved in the antimicrobial action of SNAPPs.

Next, cryo-TEM was used to visualize the effect of SNAPP treatment on E. coli cell morphology. Prior to treatment, all cells showed intact OMs and CMs (Figure 2.4a, Appendix I, Supplementary Figure 2.34a-c). After treatment with S16 in MHB at its MBC, large aggregates – probably formed by aggregation between S16 and media contents (vide supra) – were observed around the cells (Figure 2.4b-g, Appendix I, Supplementary Figure 2.34d-g). The cell membranes of bacteria incubated with S16 appeared disrupted (Figure 2.4b) and had pores that transversed the OM, PG layer, and CM (Figure 2.4c, Appendix I,
Supplementary Figure 2.34d). Most bacterial cells had fragmented or perforated OMs (Figure 2.4d-e, Appendix I, Supplementary Figure 2.34e-f), and some cells appeared to be broken into isolated fragments (Figure 2.4g, Appendix I, Supplementary Figure 2.34g). These observations were in agreement with the flow cytometry data which indicated that SNAPP association leads to membrane disruption. Cryo-TEM experiments conducted in MEM at supra-MBC dosage resulted in observations similar to when MHB was used at similar dosages; however, more drastic cell lysis was noted in the case of MEM (Appendix I, Supplementary Figure 2.35).

Figure 2.4. Morphological studies of *E. coli* before and after treatment with S16 in Mueller-Hinton broth (MHB). a-g, Cryo-TEM images of *E. coli* before (a) and after incubation with S16 for 90 min at a lethal dose of 35 µg/mL (i.e., 1 × MBC of the unlabelled S16 in MHB) (b-g). Large aggregates (possibly aggregates of S16 with medium contents) were observed around the rod-shaped *E. coli* cells (b-g). Binding of the aggregates to *E. coli* was observed and the cell membrane appeared disrupted (b). Hole formation (c), OM fragmentation (d), stripping of cell walls and membranes (e), ripping of cell ends (f), and isolated cell fragments (g) were observed. Enlarged images of a-e are provided and the sections enlarged are boxed in red. Scale bars, 200 nm, 250 nm, or 500 nm as indicated. Regions of interest are highlighted by red arrows. All images are representative of three independent experiments.
Taken together, it was postulated that SNAPPs have a multi-modal mechanism of inducing bacterial cell death. Initially, SNAPPs bind via electrostatic interactions with LPS and the OM, leading to destabilized/fragmented areas. They then assemble and traverse the cell envelope driven by the transmembrane electrical potential (interior/cytosol being negative), most likely causing membrane perturbations that result in unregulated transmembrane ion movement in the CM. These membrane disruption events induce ALD at low SNAPP concentrations, thus leading to cell lysis [42]. At high concentrations, SNAPPs rapidly cause cell lysis by direct disruption of the OM and CM. Collectively, fluorescence imaging, flow cytometry and cryo-TEM assays provided evidence for membrane association, membrane disruption, OM fragmentation/destabilization and cell lysis. Although the exact sequence of events requires further investigations, it can be concluded that the succession of antimicrobial events, as depicted in Figure 2.5, is substantially different from the action of a monomeric cationic AMP. A typical monomeric AMP is commonly thought to traverse the OM of a Gram-negative bacterial cell via self-promoted uptake, binds to and inserts itself into the anionic surface of the CM, then kills the bacteria by either membrane disruption (pore formation) or translocation across the CM and acting on internal targets [12,43,44]. In the case whereby cell death is caused by membrane disruption, loss of CM integrity was commonly thought to be the lethal event [12,45,46]. Cryo-TEM analysis of *E. coli* after incubation with melittin or ovispirin (which are well-characterized AMPs) confirmed that the AMPs disrupted the CM while leaving the OM intact (Appendix I, Supplementary Figure 2.36). On the other hand, as demonstrated here, it is likely that S16 effects its antimicrobial action in a cascade manner and by first disrupting the physical integrity of the OM upon binding with LPS. It was hypothesized that the difference in the mode of action between monomeric cationic AMPs and S16 might be attributed to the latter’s structural architecture, as SNAPPs might be unable to translocate across the OM and CM without causing significant perturbation.
Figure 2.5. A comparison between the antimicrobial mechanism(s) of typical membrane-disrupting cationic AMPs and the possible mechanism of SNAPPs against Gram-negative bacteria. (a) Cationic AMPs bind to the OM of Gram-negative bacteria via electrostatic interactions, transit across the OM through membrane destabilization, and disrupt the physical integrity of the CM by the ‘barrel-stave’, ‘toroidal-pore’ or ‘carpet’ pore (not shown in figure) mechanisms [12]. (b) SNAPPs, whether in its aggregated or unaggregated state, interact with the OM, PG and CM layers of Gram-negative bacteria via electrostatic attractions and kill the cell by fragmenting/destabilizing its OM and possibly disrupting the CM such that unregulated ion movement is resulted, but also by the induction of the apoptotic-like death pathway (not shown in figure), thereby lysing the cell.

AMPs as a whole class of compounds kill bacteria by multiple mechanisms but each specific AMP tends to kill bacteria by one major mechanism [8]. The multi-faceted interactions shown here between SNAPPs and bacteria (in combination with indirect antimicrobial activity via neutrophil recruitment in vivo) are truly unique and this has not been shown previously in a definitive manner for any one single AMP. Silver ions have been shown to enhance antibiotic activity by interacting with several cytosolic molecules, which corroborates the data that an antimicrobial agent may have more than one mode of action [47]. The equipotency of SNAPPs against all of the Gram-negative bacteria tested suggested that the multi-modal mechanism of action is non-specific, which could explain why bacteria did not acquire resistance to star S16 even after 600 generations of growth in the presence of the agent. The successful demonstration of the antimicrobial efficacy of SNAPPs against CMDR A. baumannii in vivo – the first successful in vivo attempt for any synthetic AMP mimic or antimicrobial polymer against CMDR Gram-negative bacterial strains – will undoubtedly distinguish SNAPPs as a new class of antimicrobial agents for combating infectious disease.
2.4 Chapter Summary

In conclusion, the successful design and synthesis of 16- and 32-arm star peptide polymer nanoparticles (termed ‘SNAPPs’) comprising lysine and valine residues were shown in this chapter. SNAPPs were found to efficiently kill a wide range of clinically-relevant Gram-negative bacteria, including several ESKAPE and CMDR pathogens, at sub-μM concentrations. When compared to various AMPs previously reported, SNAPPs are superior in terms of their ability to kill Gram-negative bacteria while retaining high therapeutic indices. SNAPPs also showed no observed development of resistance in A. baumannii. In addition, preliminary animal studies have shown that SNAPPs were effective against CMDR A. baumannii in vivo. With the aid of complementary imaging techniques and bioassays including super-resolution fluorescence imaging, cryo-TEM, competitive inhibition assays, bacterial membrane potential analysis as well as LUV and gene expression studies, it was postulated that the antimicrobial mechanism of SNAPPs proceeded via a multi-modal mechanism of cell death characterized by OM destabilization, CM perturbations that result in unregulated transmembrane ion movement, and induction of the ALD pathway – all leading to cell lysis. The efficient antimicrobial efficacies and biocompatibility demonstrated by the SNAPPs make them promising candidates as a new class of antimicrobial agents, potentially capable of addressing the dearth of suitable drug candidates to combat Gram-negative pathogens resistant to conventional antibiotics.

2.5 References


CHAPTER 3

Effects of Polymer Structure on the Antimicrobial Activity and Biocompatibility of Star Peptide Polymers

3.1 Chapter Perspective

In this chapter, a library of star and linear peptide polymers was designed to evaluate the influence of star arm (co)polymer structure and macromolecular architecture on antimicrobial activity against Gram-positive and negative bacteria, and biocompatibility. Using advanced flow cytometry techniques coupled with conventional antimicrobial testing methods, it was demonstrated that 16- and 32-arm amphiphilic star polymers with random copeptide arms consisting of lysine and valine residues were superior to the block copeptide and homo poly(lysine) stars, as well as their linear analogues, in terms of their abilities to disrupt the membranes of and kill both Gram-negative and positive bacteria. In a membrane-mimicking environment, only the random star peptide polymers adopted an overall $\alpha$-helical conformation, which was observed to contribute to their greater potency. All peptide polymers studied were found to be non-hemolytic, even at a high concentration of 1 mg/mL. The star polymers with random copeptide arms possess favourable therapeutic indices (126-161) and had low toxicity towards mammalian cells (IC$_{50}$ 121-128 $\mu$g/mL against HEK293T cells). These findings provided valuable insights into the structural determinants of antimicrobial star peptide polymers and will contribute towards developing the next generation of star peptide polymers with enhanced potency and selectivity.
3.2 Introduction

In Chapter 2, the discovery of a new class of antimicrobial agents, in the form of 16- and 32-arm star-shaped polymers that possess a poly(amido amine) (PAMAM) dendritic core and statistical copeptide polymer arms consisting of cationic lysine and hydrophobic valine amino acid repeat units, was presented [1]. The star peptide polymers, termed ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs), were synthesized via ring-opening polymerization (ROP) of \( \alpha \)-amino acid \( N \)-carboxyanhydrides (NCAs) through a core-first approach. SNAPPs were found to demonstrate excellent efficacy against Gram-negative bacteria without observable resistance development, including several ‘ESKAPE’ pathogens (i.e., a class of bacteria known to cause the majority of nosocomial infections) and their multidrug-resistant (MDR) clinical isolates. SNAPPs also displayed low toxicity against mammalian cells, which translated to high therapeutic indices (TIs). Compared to SNAPPs, the linear analogue (one star arm equivalent) was inferior in antimicrobial efficacy. SNAPPs were effective at combating infections caused by MDR Acinetobacter baumannii that is also resistant to colistin, which is widely regarded as the ‘last line of defense’ against MDR Gram-negative pathogens [2]. Further, SNAPPs were shown to possess a unique, multi-modal mechanism of activity that proceeds via outer membrane destabilization, unregulated ion movement across the cytoplasmic membrane, and induction of the apoptotic-like death pathway in bacteria. This mechanism differs from that commonly documented for antibiotics and AMPs, and could potentially account for why resistance to SNAPPs cannot be acquired easily.

Based on the findings presented in Chapter 2, several key scientific questions were raised, such as the relationship(s) between the polymer structure and function, i.e., (1) the effect of star arm (co)polymer structure (block copolymer or homopolymer versus random copolymer) on bacterial membrane disruption ability, antimicrobial activity and biocompatibility, and (2) the reason as to why the ‘one arm’ linear peptide polymer equivalent of SNAPPs was inferior in efficacy. In order to answer these questions and to better understand the structure-activity relationships of the peptide polymers, a library consisting of 16- and 32-arm star peptide polymers and linear peptide polymers was designed to compare newly synthesized block copeptide polymers and homopeptides with the random copeptide polymers reported in our earlier study [1]. The antimicrobial properties against Gram-positive and Gram-negative bacteria, and mammalian cell toxicity effects were
assessed using standard antimicrobial susceptibility, hemolysis and apoptosis assays. The use of a high-throughput approach based upon flow cytometry and nucleic acid staining of bacterial cells to enable the rapid screening of the membrane disrupting ability of the peptide polymers was also demonstrated. To elucidate the effect of the star architecture and the possible contribution of secondary structure on antimicrobial activity, circular dichroism (CD) spectroscopy was conducted to compare the secondary structures of the star and linear polymers under conditions that mimicked interaction with the bacterial membrane.

3.3 Results and Discussion

3.3.1 Star Peptide Polymer Design and Synthesis

A polymer library consisting of six star peptide polymers was synthesized via ROP of amino acid NCA monomers using the primary amines of multifunctional dendrimers as initiators (Figure 3.1). Lysine and valine were selected as cationic and hydrophobic amino acids, respectively. Second (G2) and third (G3) generation PAMAM dendrimers with 16 and 32 peripheral primary amines were used as initiators to efficiently prepare 16- and 32-arm star polymers, respectively [3]. The stoichiometric ratio of the NCA monomers to the initiators was controlled to target a theoretical degree of polymerization (DP) of 30 per star arm at complete monomer conversion. The monomer conversions for all star polymers were ca. 80% as observed with $^1$H NMR analysis, which yielded a DP of 24 per star arm and molecular weights ranging from 43.6 to 92.5 kDa (Table 3.1). For star polymers comprising of both lysine and valine, the mole ratio of lysine to valine was kept at 2:1, which was determined to be the optimum ratio for water solubility while resembling the typical cationic-to-hydrophobic ratio of membrane-active AMPs [4]. As reported in Chapter 2 [1], lysine and valine NCAs were randomly polymerized to form the random copeptide arms of 16- and 32-arm star polymers SR$_{16,Z}$ and SR$_{32,Z}$, respectively. Noteworthy, the reactivity ratio of lysine and valine NCAs are ca. 1 [5], thus indicating the formation of statistical copeptide polymers. To facilitate comparison with SR$_{16,Z}$ and SR$_{32,Z}$, in this study, the star arm (co)polymer structure was systematically varied to prepare four other star peptide polymers. 16- and 32-arm star polymers, SB$_{16,Z}$ and SB$_{32,Z}$, respectively, possess block copeptide arms consisting of poly(DL-valine) (PVal) at the corona and poly($\varepsilon$-Z-L-lysine) (PZLL; carboxybenzyl (Cbz or Z) protected) nearer to the core. Stars SH$_{16,Z}$ and SH$_{32,Z}$, on the other hand, are 16- and 32-arm star polymers, respectively, with PZLL homopeptide arms.
Figure 3.1. Synthesis of star peptide polymers. Synthesis of the star peptide polymers via ROP of lysine NCA only (SH$_{16,Z}$, SH$_{16}$, SH$_{32,Z}$, and SH$_{32}$) or both lysine NCA and valine NCA (SB$_{16,Z}$, SB$_{16}$, SB$_{32,Z}$, SB$_{32}$, SR$_{16,Z}$, SR$_{16}$, SR$_{32,Z}$, and SR$_{32}$) was initiated from the
terminal amines of PAMAM dendrimers. The number of repeat units for lysine and valine are $a$ and $b$, respectively. See also Table 3.1 for the lysine-to-valine ratios (i.e., $a:b$) of copolymers of lysine and valine.

**Table 3.1. Composition, Molecular Weight and Hydrodynamic Diameter of the Star and Linear Peptide Polymer Library.**

<table>
<thead>
<tr>
<th>Peptide Polymer</th>
<th>Arm Number</th>
<th>Lysine:Valine, $a:b^a$</th>
<th>Molecular Weight (kDa)$^b$</th>
<th>Hydrodynamic diameter, $D_H$ (nm)$^c$</th>
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</thead>
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<tr>
<td><strong>Star</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Block</td>
<td>SB$_{16}$</td>
<td>16</td>
<td>3.19:1</td>
<td>46.5</td>
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<tr>
<td></td>
<td>SB$_{32}$</td>
<td>32</td>
<td>2.87:1</td>
<td>92.5</td>
</tr>
<tr>
<td>Random$^d$</td>
<td>SR$_{16}$</td>
<td>16</td>
<td>1.95:1</td>
<td>45.4</td>
</tr>
<tr>
<td></td>
<td>SR$_{32}$</td>
<td>32</td>
<td>2.00:1</td>
<td>90.9</td>
</tr>
<tr>
<td>Homo</td>
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<td>-</td>
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</tr>
<tr>
<td></td>
<td>SH$_{32}$</td>
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<tr>
<td>Homo</td>
<td>LH</td>
<td>-</td>
<td>-</td>
<td>3.6</td>
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$^a$ Determined by $^1$H NMR spectroscopic analysis. $^b$ Molecular weights were calculated based on $^1$H NMR. $^c$ Determined by dynamic light scattering (DLS) analysis. $^d$ The synthesis and characterization of the polymers were reported in Chapter 2 [1].

Successful synthesis of the star peptide polymers was confirmed by $^1$H NMR spectroscopic analysis (Appendix II, Supplementary Figure 3.1). For the star peptide polymers comprised of lysine and valine (SB$_{16,Z}$, SB$_{32,Z}$, SR$_{16,Z}$ and SR$_{32,Z}$), proton resonances characteristic of valine (i.e., $\delta_H$ 0.8 ppm corresponding to the methyl groups on the valine side chain) and lysine (i.e., $\delta_H$ 1.2-1.8 ppm corresponding to the methylene protons on the lysine NCA side chain) residues were observed. Integration and comparison of these resonances provided lysine-to-valine ratios of approximately 2-3:1, which is consistent with the ratio of lysine and valine NCA monomers used in the synthesis (Appendix II, Supplementary Table 3.1). Resonances resulting from the G2 and G3 PAMAM cores were difficult to observe upon star formation as they overlap with the broad peptide polymer peaks [6]. It is also well-known that the core of star polymers lacks mobility and hence is not typically detected in NMR spectroscopy [7]. The subsequent removal of the carboxylbenzyl (Cbz or Z) protecting groups on the lysine residues along the arms of the star peptide polymers using HBr yielded water-soluble stars (SB$_{16}$, SB$_{32}$, SR$_{16}$, SR$_{32}$, SH$_{16}$, and SH$_{32}$) with pendent protonated amine
functionalities along the arms. At physiological pH, the pendent amine groups (pKa = 10.5) would remain protonated. $^1$H NMR spectroscopic analysis revealed quantitative deprotection (i.e., > 99% removal of the Cbz protecting groups) for all of the polymers (Appendix II, Supplementary Figure 3.2). The lysine-to-valine ratios of the star peptide polymers remained similar after deprotection (ca. 2-3:1; Table 3.1), indicating that the polymeric structures were not altered by the deprotection process.

In order to characterize the solvated dimensions of the star peptide polymers, dynamic light scattering (DLS) analysis was conducted in water. The particle size distribution of each polymer was monomodal with hydrodynamic diameters ($D_H$) that range from 7.5 to 16.8 nm (Table 3.1 and Appendix II, Supplementary Figure 3.3). Using aqueous gel permeation chromatography, the molecular weight characteristics of all deprotected star peptide polymers were determined relative to poly(ethylene glycol) (PEG) standards (Appendix II, Supplementary Table 3.1). Further attempts to analyze the star peptide polymers via MALDI-ToF spectrometry proved futile as the molecular weights are beyond the detection limit of this analytical technique to obtain any reasonable spectra.

### 3.3.2 Antimicrobial Properties of Star Peptide Polymers

The antibacterial efficacy of the star peptide polymers against *Escherichia coli* and *Staphylococcus aureus* – commonly used model Gram-negative and positive bacteria, respectively – is presented in Table 3.2. The antimicrobial activities were assessed in nutrient-rich media (i.e., undiluted Mueller-Hinton broth, MHB, for *E. coli* and Luria broth, LB, for *S. aureus*) to ensure optimum bacterial growth. Firstly, as it was demonstrated in Chapter 2 that the bactericidal action of SNAPPs involved membrane disruption, the ability of the star peptide polymers synthesized herein to disrupt bacterial membranes was evaluated via determination of their minimum membrane disruptive concentrations (MDCs). The MDC is defined as the minimum drug (in this case, peptide polymer) concentration that causes membrane disruption in all cells, and was determined using a combination of nucleic acid staining and a high throughput flow cytometric analytical protocol as previously reported [8]. The MDC assay is a rapid and quantitative method that enables the assessment of antimicrobial activities by measuring peptide-bacteria interactions and lysed cell numbers. In this chapter, antimicrobial properties were also evaluated using traditional antimicrobial susceptibility testing methods, namely the minimum bactericidal concentration (MBC), which is defined as the minimum drug concentration that causes quantitative bacterial cell death based on colony-forming unit (CFU) measurements, and the minimum inhibitory
concentration (MIC), which is a turbidity-based measurement of the lowest drug concentration required to result in quantitative bacterial growth inhibition (see Experimental Procedure). Compared to these growth-based MBC and MIC assays that require at least an overnight incubation, the use of flow cytometry enables MDC determination to be completed within 90 min.

Table 3.2. MDCs and MBCs of the Star and Linear Peptide Polymers against *E. coli* and *S. aureus*.

<table>
<thead>
<tr>
<th>Peptide Polymer</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDC&lt;sup&gt;a&lt;/sup&gt; (µM)</td>
<td>MBC&lt;sup&gt;b&lt;/sup&gt; (µM)</td>
</tr>
<tr>
<td><strong>Star</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Block</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB&lt;sub&gt;16&lt;/sub&gt;</td>
<td>17.3 ± 1.3</td>
<td>10.5 ± 4.7</td>
</tr>
<tr>
<td>SB&lt;sub&gt;32&lt;/sub&gt;</td>
<td>6.2 ± 0.1</td>
<td>6.1 ± 1.3</td>
</tr>
<tr>
<td>Random&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>SR&lt;sub&gt;16&lt;/sub&gt;</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>SR&lt;sub&gt;32&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homo</td>
<td>3.0 ± 1.0</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>SH&lt;sub&gt;16&lt;/sub&gt;</td>
<td>2.0 ± 0.1</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>SH&lt;sub&gt;32&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Linear</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11.4 ± 2.9</td>
<td>29.5 ± 0.1</td>
</tr>
<tr>
<td>Homo</td>
<td>27.5 ± 0.6</td>
<td>13.4 ± 1.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> MDC is defined as the minimum polymer concentration that causes membrane disruption in all cells. <sup>b</sup> MBC is defined as the minimum polymer concentration that causes 100% bacterial cell death. <sup>c</sup> MIC is defined as the minimum polymer concentration that causes microbial growth inhibition. <sup>d</sup> The MBC data of the indicated polymers were reported in Chapter 2 [1].

Compared to the other star peptide polymers within the library, star SR<sub>16</sub> with random copeptide arms was the most effective at causing membrane disruption, with MDCs of 0.8 and 1.0 µM against *E. coli* and *S. aureus*, respectively. By comparing these values to those of several common AMPs, such as ovispirin, magainin II, alamethicin, and caerin 1.1, SR<sub>16</sub> was found to possess higher membrane disrupting abilities by at least one order of magnitude [8]. On the other hand, star polymer SB<sub>16</sub> with block copeptide arms registered the highest MDC (i.e., lowest membrane disrupting ability) against *E. coli* compared to the star polymers with random copeptide arms and homopeptide PLL arms. Furthermore, within the concentration
range tested (≤ 100 µM), the star peptide polymer SB\textsubscript{16} did not show any significant disruptive activity towards the membranes of \textit{S. aureus}. This implied that the block configuration, which consists of a hydrophobic, outer peptide block and an inner, cationic peptide block, is not favourable for bacterial membrane disruption. Homopeptide star SH\textsubscript{16} with cationic arms was more effective than block copeptide star SB\textsubscript{16} in disrupting the membranes of \textit{E. coli} and \textit{S. aureus}, but was inferior to the random copeptide star SR\textsubscript{16}. Similar trends were also observed for the 32-arm star peptide polymers (Table 3.2). These results elucidate the importance of both peptide composition and sequence in dictating the membrane lysis abilities of these macromolecular antimicrobials. Specifically, it was postulated that the presence of both unshielded cationic and hydrophobic segments, such as that provided by a random copolymer configuration, is necessary for microbial membrane disruption. While stars having block copeptide arms with PLL on the corona and PVal nearer to the core might display membrane lytic properties comparable to or even better than those of stars SR\textsubscript{16} and SR\textsubscript{32}, precipitation of the PVal star (due to aggregation as a result of β-sheet formation [9]) prior to addition of the second block rendered the synthesis of these block copeptide stars challenging.

The MDCs of the star peptide polymers were compared to their MBCs and MICs (Table 3.2) to provide more comprehensive insights on the effect(s) of the peptide polymers on \textit{E. coli} and \textit{S. aureus}. When tested against \textit{E. coli}, the MBCs and MICs of stars SB\textsubscript{16}, SB\textsubscript{32}, SR\textsubscript{32} and SH\textsubscript{16} were in agreement with their respective MDCs. This indicated that membrane disruption (as indicated by the MDC) and cell death (as indicated by the MBC and suggested by the MIC) occurred simultaneously for the aforementioned peptide polymers. From the antibacterial tests against \textit{S. aureus}, star polymers SB\textsubscript{16} and SB\textsubscript{32} displayed no MBCs or MICs, which was consistent with their inability to disrupt the membrane of \textit{S. aureus}.

The star peptide polymer SH\textsubscript{32} was found to be bactericidal against \textit{E. coli}; interestingly its MDC of 2.0 µM was much higher than its MBC of 0.9 µM and MIC of 0.8 µM. These differences indicate that membrane disruption was not the primary mechanism involved in the killing of \textit{E. coli} by polymer SH\textsubscript{32}. As previously shown in Chapter 2, the production of reactive oxygen species (ROS) and the induction of apoptotic-like death (ALD) responses were supplementary bactericidal mechanisms of SR\textsubscript{16}, aside from its primary mode of action - membrane disruption [1]. In the case of star SH\textsubscript{32}, it was postulated that the supplementary mechanisms could dominate, resulting in the ability of the star to effect cell death even before quantitative membrane disruption. Further, the existence of other
antibacterial mechanisms was also possible, such as the inhibition of cellular processes through interactions with other intracellular enzyme or nucleic acid targets [10]. Interestingly, homopeptide stars $\text{SH}_{16}$ and $\text{SH}_{32}$, which were able to quantitatively disrupt the membrane of $S. \text{aureus}$, did not register any MBC or MIC against $S. \text{aureus}$ within the range of concentrations tested. Furthermore, polymer $\text{SR}_{16}$, which was the most effective star polymer in terms of membrane disruption, had an MBC against $S. \text{aureus}$ that was higher than its MDC. A possible explanation is that while these polymers were capable of disrupting bacterial membranes, membrane disruption itself was not sufficient to induce quantitative death in $S. \text{aureus}$. In fact, it is likely that the membrane pores induced by these polymers had low overall stability and half-lives, thereby allowing the fluid lipid bilayers of the bacterial membrane to reorganize and recover from the initial disruptive effects of the polymers [11]. Hence, in the case of $\text{SR}_{16}$, a polymer concentration higher than its MDC ($i.e.$, MBC 4.6 μM) was needed for complete cell death of $S. \text{aureus}$. Taken together, these results indicated the complex nature of the antibacterial mechanism of the star peptide polymers studied due to the multiple targets involved, which highlighted the importance of performing in-depth mechanistic studies (such as evaluating polymer-membrane interactions through MDC determination as shown) to better understand the structure-activity relationships of antimicrobial polymers in general.

The poor correlation noted between the MICs of several polymers and their respective MBCs and/or MDCs (such as $\text{SR}_{16}$ against $E. \text{coli}$ and $\text{SR}_{32}$ against $S. \text{aureus}$) implied that MIC measurements might not be a reliable technique for this class of polymers, possibly due to the interactions between the polymers and medium contents. As an extended duration of incubation was required in MIC assays, the precision of the turbidity-based MIC measurements could be affected by polymer aggregation as a result of interactions with the nutrient broth components ($e.g.$, proteins). The formation of aggregates with medium contents has been observed for $\text{SR}_{16}$ in our previous work [1]. This was also observed by Oda \textit{et al.} and Yang \textit{et al.} for cationic polymers, where the plate killing assay (MBC) was selected as the method of choice to evaluate the efficacy of antimicrobial polymers over turbidity-based assays (MIC) [10,12]. Further, MBC and MDC assays, which measure bacterial cell death/lysis, are able to provide more conclusive insights on the antibacterial activities of the peptide polymers compared to the MIC assay, which evaluates bacterial growth inhibition. Based upon the MDCs and MBCs, it can be concluded that the star polymers with random copeptide arms ($\text{SR}_{16}$ and $\text{SR}_{32}$) were superior to other star polymers in the library.
displaying excellent activity against *E. coli* and satisfactory potency against *S. aureus*. Overall, all star variants were also found to be more effective against *E. coli* than *S. aureus*. This agreed with the findings presented in Chapter 2 where antimicrobial star peptide polymers (SNAPPs) were shown to have a stronger and more preferential binding affinity to the lipopolysaccharide layer found only on the outer membrane of Gram-negative bacteria [1].

### 3.3.3 Effect of the Star Architecture on the Antimicrobial Efficacy of Peptide Polymers

To investigate the effect of the star architecture on antibacterial activity, linear homo (**LH**) and random (**LR**) peptide polymer analogues were prepared for comparison. While peptide polymer **LH** was synthesized to represent one arm of stars **SH**₁₆ and **SH**₃₂, the peptide polymer **LR** is the linear analogue (one arm equivalent) of stars **SR**₁₆ and **SR**₃₂. It should be noted that linear counterparts of star peptide polymers **SB**₁₆ and **SB**₃₂ were not synthesized as the linear amphiphilic block copolymers could possibly self-assemble or aggregate in solution, making them unreliable for any systematic comparison. Further, the preparation of linear, amphiphilic peptide polymers with similar molecular weights as the stars (>500 repeat units needed) was not possible synthetically, which highlighted the advantage of the star polymer platform in synthesizing high molecular weight, stable and covalently-linked peptides that are hard to access via other methods. Details describing the synthesis and characterization of the linear peptide polymers are provided in Table 3.1 and Appendix II, Supplementary Figures S4 and S5 and Supplementary Table 3.2.

The antibacterial properties of the linear peptide polymers are provided in Table 3.2. The amphiphilic star peptide polymers **SR**₁₆ and **SR**₃₂ possessed efficacy against both *E. coli* and *S. aureus*, while their linear analogue **LR** was poorly active with an MBC that was 40-fold higher. By evaluating the MDCs and MICs, it was validated that **LR** displayed weak antibacterial action, both in terms of bacterial membrane disruption and growth inhibition, with an MDC that was 14-fold higher than those of the stars and no MIC recorded within the range of concentrations tested (≤ 100 µM). In the case of the purely cationic PLL polymers, the star polymers **SH**₁₆ and **SH**₃₂ were found to exhibit enhanced antimicrobial activity compared to the linear analogue **LH**, albeit to a lesser extent (5 to 15-fold in terms of the MBCs) compared to the random copeptide polymers.

To investigate whether this observation applies to other bacteria, antimicrobial testing was conducted on another bacterial species, *Streptococcus mutans*. As expected, the amphiphilic star peptide polymers **SR**₁₆ and **SR**₃₂ displayed good bactericidal activity (MBC
while LR was ineffective (Appendix II, Supplementary Table 3.3). These results strongly suggested that the star architecture had a significant effect on enhancing the antimicrobial effect of peptide polymers, especially random copeptide polymers, against a range of Gram-positive and Gram-negative bacteria.

To further investigate the rationale behind this observation, CD spectroscopy was used to assess the secondary structure of the star and linear peptide polymers in water with incremental additions of trifluoroethanol (TFE). TFE, which is a well-known secondary structure inducer, is commonly used to mimic the hydrophobic cell membrane environment [13-15]. By progressively increasing the amount of TFE in an aqueous environment, we investigated the propensity of the peptide polymers to form distinct secondary structure. At 0, 20 and 50% v/v TFE, none of the peptide polymers exhibited any distinct secondary structures (Appendix II, Supplementary Figure 3.6a-c). Interestingly, when the amount of TFE was increased to 80% v/v, SR16 and SR32 displayed distinct spectra characteristic of α-helices (Figure 3.2). The linear analogue of these star peptide polymers (i.e., LR), on the other hand, displayed only a very weak (almost negligible) α-helical character as indicated by the low intensity of the spectrum relative to those of SR16 and SR32 (Figure 3.2). No distinct secondary structure was observed for any of the other peptide polymers (Appendix II, Supplementary Figure 3.6d). The CD spectroscopy results demonstrated that the star peptide polymers SR16 and SR32 had the propensity to adopt α-helical structures in a hydrophobic environment (mimicked by the 80% v/v TFE solution), and indicated a strong correlation between α-helicity and the excellent antibacterial activities of the polymers against E. coli, S. aureus, and S. mutans. It was therefore hypothesized that the star architecture amplified the α-helical character of the random copeptide polymers when in contact with bacterial membranes, which possibly contributed to the excellent potency of SR16 and SR32 against bacteria compared to their linear analogue. It is well known that the most abundant class of AMPs adopts an α-helical conformation in hydrophobic environments and helicity has been demonstrated in some studies to be crucial for the antimicrobial activity of these peptides [16,17]. Noteworthy, the concentration of the peptide polymers used in CD spectroscopy experiments was at an optimized value of 0.2 mg/mL, which is above the MBC and MIC values of the compounds.
Figure 3.2. CD spectra of SR\textsubscript{16}, SR\textsubscript{32} and LR (at a concentration of 0.2 mg/mL) in RO water with 80\% v/v TFE. The arrows point towards the troughs on the spectra for SR\textsubscript{16} and SR\textsubscript{32}, which are characteristic of $\alpha$-helices. Arrows with solid line: Characteristic troughs between 205 and 210 nm. Arrows with dotted line: Characteristic troughs at 220 nm. Inset: Expanded view of the CD spectra of LR.

While the star architecture (from a peptide valency of 1 to 16) was found to enhance antimicrobial activity, the results herein suggested that increasing the number of star arms from 16 to 32 did not result in any substantial improvement in antimicrobial efficacy, and no obvious trends in MDCs, MBCs and MICs could be detected. This was supported by Young \textit{et al.} who reported that bacterial killing by AMPs is not necessarily proportional to peptide valency (which is represented by the number of star arms in this case) [18]. A thorough elucidation of the relationship between peptide valency (number of star arms) and antimicrobial efficacy would require further investigations, such as expanding the library of star polymers through the addition of a series of star peptide polymers with varying number of star arms (\textit{e.g.}, from 2 to 15).

\textbf{3.3.4 Peptide Polymer Biocompatibility}

As an initial test of biocompatibility, the hemolytic activities of all peptide polymers (which are their abilities to lyse red blood cells (RBCs)) were assessed by incubating them
with RBCs at different peptide polymer concentrations. While the star architecture and peptide (co)polymer structure were shown to influence antimicrobial activity significantly, these factors did not seem to have an effect on the hemocompatibility of the peptide polymers. Minimal hemolysis was demonstrated for all the peptide polymers used in this study and even at a high concentration of 1 mg/mL, the extent of hemolysis was well below 50% (Figure 3.3 and Table 3.3). This is in contrast to other synthetic polymers that reported good antimicrobial efficacy but also possess high hemolytic activity [19]. By comparing their MBCs against *E. coli* to their hemolytic activities (based on extrapolated HC$_{50}$, i.e., the 50% hemolytic concentration), the peptide polymers displayed favourable TIs (determined as HC$_{50}$/MBC$_{50}$), with the highest indices shown by SR$_{16}$, SR$_{32}$, SH$_{32}$, and LH (i.e., 126-223) (Table 3.3). When compared to the TIs of several AMPs known to be effective against Gram-negative bacteria, including magainin II, ovispirin and melittin (TIs 0.2-15, Appendix II, Supplementary Table 3.4), and multivalent polymer-AMP conjugates (TIs 11-17, Appendix II, Supplementary Table 3.4), the peptide polymers developed in this study demonstrated higher selective toxicity against bacteria. The low hemolytic activities of the peptide polymers, despite having strong antibacterial properties, could be attributed to the fact that the RBC membranes comprise neutral outer leaflets and slightly negatively charged inner leaflets at physiological pH, while bacterial membranes are highly negatively charged [20]. Consequently, the electrostatic interaction between the cationic peptide polymers and the RBC membranes was not as strong as that between the peptide polymers and the bacterial membranes.
Figure 3.3. Percent hemolysis as a function of peptide polymer concentration. Data are represented as mean ± standard deviation (SD) \((n = 4)\).

Table 3.3. Biocompatibility of Star and Linear Peptide Polymers (Determined by Hemolysis (HC\(_{50}\)) and YO-PRO-1/PI Apoptosis (IC\(_{50}\)) Assays).

<table>
<thead>
<tr>
<th>Peptide polymer</th>
<th>HC(_{50}) (a), (\mu g/\text{mL} \ (\mu M))</th>
<th>HC(<em>{50})/MBC(</em>{50}) (b) (E. coli)</th>
<th>IC(_{50}) (c), (\mu g/\text{mL} \ (\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Star</strong></td>
<td></td>
<td></td>
<td>HEK293T</td>
</tr>
<tr>
<td>Block</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB(_{16})</td>
<td>6110 (555.5)</td>
<td>105</td>
<td>&gt;128.0</td>
</tr>
<tr>
<td>SB(_{32})</td>
<td>1850 (121.7)</td>
<td>40</td>
<td>&gt;128.0</td>
</tr>
<tr>
<td>Random(d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR(_{16})</td>
<td>2550 (58.3)</td>
<td>161</td>
<td>121.8 (2.8)</td>
</tr>
<tr>
<td>SR(_{32})</td>
<td>3390 (45.3)</td>
<td>126</td>
<td>128.0 (1.7)</td>
</tr>
<tr>
<td>Homo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH(_{16})</td>
<td>3300 (119.6)</td>
<td>82</td>
<td>&gt;128.0</td>
</tr>
<tr>
<td>SH(_{32})</td>
<td>5330 (94.7)</td>
<td>223</td>
<td>&gt;128.0</td>
</tr>
<tr>
<td><strong>Linear</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random(d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LR</td>
<td>5194 (1060.0)</td>
<td>72</td>
<td>&gt;128.0</td>
</tr>
<tr>
<td>Homo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td>3071 (853.1)</td>
<td>127</td>
<td>&gt;128.0</td>
</tr>
</tbody>
</table>

\(a\) HC\(_{50}\) is the peptide concentration which results in 50\% hemolysis. Since the hemolytic activities of the star and linear peptide polymers at the highest concentration tested (1 mg/mL) were below 50\%, their HC\(_{50}\) values were extrapolated from Figure 3.3. \(b\) MBC\(_{50}\) is the peptide concentration which results in 50\% bacterial cell death. \(c\) IC\(_{50}\) is the peptide concentration...
which results in 50% mammalian cell death. The HC$_{50}$ and IC$_{50}$ data of the indicated polymers were reported in Chapter 2 [1].

Subsequently, the viability of two types of mammalian cells, human embryonic kidney (HEK293T) cells and rat hepatoma (H4IIE) cells, in response to the peptide polymers was investigated. The adhered cells were incubated with the peptide polymers for 90 minutes, stained with YO-PRO-1 and propidium iodide (PI) dyes, and analyzed by flow cytometry. Using YO-PRO-1 as an indicator of early apoptosis and PI as a measure of necrosis or cell death, viable cells were determined as cells that were negative for both YO-PRO-1 and PI. The peptide polymers were found to induce both apoptosis and necrosis, but to varying extents. When tested against HEK293T cells, star peptide polymers SR$_{16}$ and SR$_{32}$ were found to have IC$_{50}$ values (i.e., peptide concentrations that result in 50% cell death) of 121.8 μg/mL (2.8 μM) and 128 μg/mL (1.7 μM), respectively (Table 3.3). However, for the other peptide polymers, cell viability remained relatively high (i.e., at least 80%) even at the highest concentration tested (i.e., 128 μg/mL) (Figure 3.4a). Against H4IIE cells, the peptide polymers are slightly more cytotoxic, with SR$_{16}$, SR$_{32}$, SH$_{32}$, and LH found to reduce the cell viability by more than 50% at 128 μg/mL (Figure 3.4b and Table 3.3). The cell line-dependent cytotoxic effects shown here were not unexpected, as similar observations have been reported for other polymeric nanoparticles and were attributed to the differences in resistance between different cell lines [21]. It is interesting to note that while star peptide polymers SR$_{16}$ and SR$_{32}$ were found to have excellent antibacterial efficacies compared to the other peptide polymers, they displayed toxicity towards mammalian cells that was higher than most of the other peptide polymers as well. In spite of this, against certain bacterial species such as E. coli, these star peptide polymers were bactericidal at concentrations which were a few times (i.e., 4 to 8 times) lower than those needed to kill mammalian cells. However, it should be noted that different environmental conditions were used in determining the IC$_{50}$ and MBC$_{50}$ values, where the media used for the antimicrobial susceptibility tests had higher protein contents than that used in determining IC$_{50}$. As shown in Chapter 2, an increase in antimicrobial efficacy was observed when the antimicrobial assays were conducted in minimal essential medium (MEM) used for mammalian cell growth [1]. Hence, by comparing the MBC$_{50}$ values determined in MEM to the IC$_{50}$ values, SR$_{16}$ and SR$_{32}$ were found to possess TIs (IC$_{50}$/MBC$_{50}$) that ranged from 16 to 57 (Appendix II, Supplementary Table 3.5). These indices are more favourable compared to a range of commercial pharmaceutical drugs, such as digoxin (cardiac drug, TI 2-3) [22], fluconazole (antifungal drug, TI ca. 10 against certain fungal strains), amphotericin (antifungal drug, TI ca. 16.
against certain fungal strains) [23], gentamicin, and polymyxin B (antibiotics, TI < 10 against certain bacterial strains) [24].

Figure 3.4. Percent viable HEK293T (a) and H4IIE (b) cells as a function of peptide polymer concentration. Viable cells are defined as cells which were PI- and YO-PRO-1-negative. Data are represented as mean ± standard deviation (SD) (n = 4).
3.4 Chapter Summary

In conclusion, a library of peptide polymers, consisting of 16- and 32-arm star polymers with different peptide arrangements along the arms, as well as their linear analogues, was successfully synthesized and characterized. Combining advanced flow cytometry techniques with traditional antimicrobial susceptibility assays, the antimicrobial properties of the peptide polymers were determined. Among the star peptide polymers, stars with random copolypeptide arms of lysine and valine were found to efficiently disrupt the membranes and kill model Gram-positive and Gram-negative bacteria, with superior performance compared to the block copolypeptide and homolysine stars. Based on secondary structure analysis, the enhancement in antimicrobial efficacy observed by the star peptide polymers with random copolypeptide arms was possibly attributed to their ability to adopt α-helical conformations in the presence of hydrophobic environments. Importantly, no significant hemolytic activity was observed for the peptide polymers over a wide range of concentrations, regardless of peptide polymer composition and copolymer structure. Although the random copolypeptide star polymers induced greater toxicity towards mammalian cells compared to the other peptide polymers studied, their therapeutic indices were still found to be favourable as a result of their excellent antimicrobial activity. This study highlights the potential of the star architecture with random copolypeptide arms as a new design motif to develop highly potent but safe antimicrobial peptide polymers that could potentially kill a wide range of multi-drug resistant and clinically significant bacteria.

3.5 References


A Bio-Nano Interaction Study on Antimicrobial Star-Shaped Peptide Polymers

4.1 Chapter Perspective

This chapter presents a detailed bio-nano interaction study on SNAPPs by assessing their antimicrobial activities against several Gram-negative bacteria in complex biological matrices. Simulated body fluid and animal serum were used as test media to reveal factors that influence the antimicrobial efficacy of SNAPPs. With the exception of *Acinetobacter baumannii*, the presence of divalent cations at physiological concentrations reduced the antimicrobial efficacy of SNAPPs from minimum inhibitory concentrations (MICs) within the nano-molar range (40-300 nM) against *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* to 0.6-4.7 µM. By using *E. coli* as a representative bacterial species, it was demonstrated that the reduction in activity was due to a decrease in the ability of SNAPPs to cause outer and cytoplasmic membrane disruption. This effect could be reversed through co-administration with a chelating agent. Interestingly, the potency of SNAPPs against *A. baumannii* was retained even under high salt concentrations. The presence of serum proteins was also found to affect the interaction of SNAPPs with bacterial membranes, possibly through intermolecular binding. Collectively, this study highlighted the need to consider the possible interactions of (bio)molecules present *in vivo* with any new antimicrobial agent under development. The results also demonstrated that outer membrane disruption/destabilization is an important but hitherto under-recognized target for the antimicrobial action of peptide-based agents, such as antimicrobial peptides (AMPs). Overall, the findings presented in this chapter could aid in the design of more efficient peptide-based antimicrobial agents with uncompromised potency even under physiological conditions.
4.2 Introduction

Several studies have suggested that while AMPs demonstrate high bactericidal activity in vitro under appropriate conditions, their potency is often antagonized under physiological conditions [1-3]. This has been the case with many newly discovered antimicrobials, such as AMPs and polymers that are designed to be antimicrobial by mimicking the structure and function of AMPs. Their actual antimicrobial efficacies were often misrepresented as the antimicrobial susceptibility tests were performed in artificial medium that promotes bacterial growth, but does not possess physiologically relevant concentrations of salt and serum [4]. The limited studies conducted so far have shown that salts (divalent cations, in particular) and serum proteins present in vivo have significant effects on the activities of nano-sized antimicrobial agents [5-9], where efficacy could be reduced due to the masking of active functional groups or enhanced bacterial outer membrane (OM) stability. Hence, an evaluation of antimicrobial efficacy in different media is imperative for a more comprehensive understanding of how bacteria and antimicrobials interact under in vivo conditions. Noteworthy, while the significance of bio-nano interactions is widely acknowledged in the mammalian cell recognition and/or uptake of nanoparticles [10-14], studies to elucidate nanoparticle-bacteria interactions in a biologically relevant environment have been overlooked [15].

In this chapter, the bio-nano interactions between SNAPPs and biological molecules, and the effects of such interactions on the antibacterial efficacy and mechanism of SNAPPs were investigated. To the best of our knowledge, such studies are rare in the field of antimicrobial research and no study has been reported on polymer-based macromolecular antimicrobials, as the few investigations that have been conducted in this area were mostly focused on antibiotics [16-20] and linear AMPs [21-23]. This study was achieved by assessing the antimicrobial efficacy of SNAPPs in media that mimic the ionic and protein composition in vivo. Specifically, SNAPPs were tested against four different Gram-negative bacterial species in simulated body fluid (SBF) – a solution with an ion concentration similar to that in blood plasma – and animal serum, which contains physiologically relevant salt and protein concentrations. The effects of polymer-medium interactions on the bactericidal action of SNAPPs were further probed by using OM and cytoplasmic (CM) permeability assays based on fluorometric assessment and flow cytometry. Based on the observations, a strategy to improve the effectiveness of SNAPPs against bacteria under physiological conditions was demonstrated.
4.3 Results and Discussion

16- and 32-arm SNAPPs $S_{16}$ and $S_{32}$, respectively, were synthesized via ring-opening polymerization (ROP) of $\alpha$-amino acid (i.e., lysine and valine) N-carboxyanhydride (NCA) monomers as reported in Chapter 2 (see Figure 4.1 for synthetic scheme) [24,25]. SNAPPs $S_{16}$ and $S_{32}$ were found to have hydrodynamic diameters ($D_H$) of 7.7 and 13.5 nm, respectively, in phosphate-buffered saline (PBS, pH 7.4), and number-average molecular weights ($M_n$) of 43.8 and 74.8 kDa, respectively (Table 4.1). The minimum inhibitory concentrations (MICs) of SNAPPs were evaluated against a range of Gram-negative bacteria that include *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*. *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* are referred to as the ‘ESKAPE’ pathogens, which are responsible for a substantial percentage of hospital-acquired infections and are capable of acquiring antibiotic resistance rapidly [26]. The MICs were firstly determined in minimal essential medium (MEM), which is a chemically-defined minimal medium supplemented with minimum non-essential amino acids and glucose (Table 4.2). The MICs of $S_{16}$ and $S_{32}$ were found to approximate their minimum bactericidal concentrations (MBCs) (Table 4.2, as previously reported [24]), indicating that SNAPPs were bactericidal. It is noteworthy that the antimicrobial activities of both $S_{16}$ and $S_{32}$ were not species-specific for the range of bacteria tested, as their MICs against different bacterial species were within a similar order of magnitude.
Figure 4.1. Synthetic scheme of SNAPPs S16 and S32. Synthesis of SNAPPs via ring-opening polymerization (ROP) of lysine and valine N-carboxyanhydrides (NCAs) was initiated from the terminal amines of poly(amido amine) (PAMAM) dendrimers.

Table 4.1. Composition, Molecular Weights and Size of SNAPPs.

<table>
<thead>
<tr>
<th>SNAPP</th>
<th>Arm number</th>
<th>Actual lysine:valine, a:b(^a)</th>
<th>Number-average molecular weight, (M_n) (kDa)(^b)</th>
<th>Weight-average molecular weight, (M_w) (kDa)(^b)</th>
<th>Hydrodynamic diameter, (D_H) (nm)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S16</td>
<td>16</td>
<td>1.83:1</td>
<td>43.8</td>
<td>127.9</td>
<td>7.7</td>
</tr>
<tr>
<td>S32</td>
<td>32</td>
<td>1.97:1</td>
<td>74.8</td>
<td>141.1</td>
<td>13.5</td>
</tr>
</tbody>
</table>

\(^a\) Determined by \(^1\)H Nuclear Magnetic Resonance (NMR) spectroscopic analysis. \(^b\) The \(M_n\), \(M_w\) and \(D\) values were determined by aqueous gel permeation chromatography (GPC) using a conventional calibration with poly(ethylene glycol) standards.
Table 4.2. Antimicrobial Activity of SNAPPs against a Range of Gram-Negative Pathogens Tested in Different Media.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>SNAPP</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt; (µM)</th>
<th>MBC&lt;sup&gt;b&lt;/sup&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MEM</td>
<td>SBF</td>
</tr>
<tr>
<td>E. coli</td>
<td>S16</td>
<td>0.17 ± 0.01</td>
<td>3.43 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>S32</td>
<td>0.05 ± 0.00</td>
<td>1.68 ± 0.07</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>S16</td>
<td>0.08 ± 0.05</td>
<td>1.64 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>S32</td>
<td>0.04 ± 0.01</td>
<td>0.59 ± 0.18</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>S16</td>
<td>0.30 ± 0.10</td>
<td>4.68 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>S32</td>
<td>0.21 ± 0.01</td>
<td>1.92 ± 0.14</td>
</tr>
<tr>
<td>A. baumannii</td>
<td>S16</td>
<td>0.13 ± 0.05</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>S32</td>
<td>0.08 ± 0.02</td>
<td>0.10 ± 0.00</td>
</tr>
</tbody>
</table>

<sup>a</sup> MIC is defined as the minimum drug concentration that causes quantitative bacterial growth inhibition.  
<sup>b</sup> MBC is defined as the minimum drug concentration that causes quantitative bacterial cell death. All MIC and MBC data are expressed as mean and standard deviations of four replicates (n = 4) completed in two independent experiments.

4.3.1 Antimicrobial Studies in Simulated Body Fluid (SBF)

Although MEM is able to sustain bacterial viability for antimicrobial susceptibility testing purposes, its ionic composition does not reflect the type and concentration of salts in vivo. For example, divalent cations such as magnesium (Mg<sup>2+</sup>) and calcium (Ca<sup>2+</sup>) ions, which are present in the blood, are absent in MEM. Therefore, to better reflect the therapeutic efficacy of SNAPPs in vivo, the MICs of the peptide polymers were evaluated in SBF supplemented with amino acids and glucose (see Chapter 7.7 for composition). The ionic composition of SBF mimics that of human blood plasma, containing Mg<sup>2+</sup> and Ca<sup>2+</sup> ions at physiological concentrations [18]. Interestingly, significant reductions (P<0.05) in inhibitory activity (as indicated by the increase in MIC values) by at least 9 times from nM to low µM MICs for both S16 and S32 against E. coli, P. aeruginosa and K. pneumoniae were observed when SBF was used as the medium, instead of MEM (Table 4.2). The antimicrobial activity of S32 against E. coli was the most severely antagonized compared to the other polymer-
bacteria combinations, as the MIC of S32 against E. coli increased more than 30 times from 0.05 µM in MEM to 1.68 µM in SBF.

It was hypothesized that the reductions in activity observed were a result of the antagonistic effects exhibited by one or more of the salts in SBF towards SNAPPs. To validate this hypothesis, modified versions of SBF were prepared in the same way as SBF but with certain salts (such as magnesium and/or calcium salts) removed. SNAPP S16 was chosen as the model compound for subsequent studies to serve as a continuation of Chapter 2, where all mechanistic studies in bacteria culture media were performed on S16. The MICs of S16 against E. coli were found to be 1.62 ± 0.17 µM in the absence of a magnesium salt (MgCl₂·6H₂O) and 0.39 ± 0.01 µM in the absence of a calcium salt (CaCl₂), which in both cases were lower than its MIC in SBF. The removal of Ca²⁺ ions resulted in a more substantial recovery in inhibitory activity, although the resulting MIC was still more than double of that in MEM. In modified SBF without both MgCl₂·6H₂O and CaCl₂ (hereby denoted as ‘mSBF’), the MIC of S16 dropped to 0.22 ± 0.03 µM, which was comparable to its activity in MEM (i.e., 0.17 µM). Similarly, the MIC of S16 against P. aeruginosa was reduced by almost 12-fold from 1.64 µM in SBF to 0.14 ± 0.06 µM in mSBF, close to its MIC in MEM.

These observations implied an antagonistic effect of both Mg²⁺ and Ca²⁺ divalent cations on the antimicrobial activity of SNAPPs. Previous studies on cationic AMPs have reported similar observations whereby the activity of certain AMPs was attenuated at high divalent cation concentrations [3]. It was postulated that divalent cations such as Mg²⁺ and Ca²⁺ bind to polyphosphates in the LPS layer of the bacterial OM, thereby stabilizing the LPS structure and reducing the overall negative charge of the OM [27-31]. Hence, the presence of these cations at high concentrations could reduce the membrane disrupting ability of cationic antimicrobial agents such as AMPs and SNAPPs, as demonstrated in this study. It was also observed that the activity of S16 was inhibited to a greater extent by Ca²⁺ than Mg²⁺ ions. This agreed with the observation made by Sahalan et al. where Ca²⁺ ions were found to be more effective than Mg²⁺ ions in deactivating polymyxin B, a cyclic cationic antimicrobial [32]. The authors postulated that Ca²⁺ ions play a more significant role in stabilizing bacterial OM than Mg²⁺ ions. Interestingly, in this study, the antagonistic effect displayed by Mg²⁺ and Ca²⁺ seemed to be species-dependent. While the antimicrobial activity of S16 and S32 against E. coli, K. pneumoniae and P. aeruginosa were affected, their activity against A. baumannii remained unchanged regardless of the salt composition of the medium used. It has
been widely acknowledged that a large diversity exists in the composition of bacterial membranes across the different species and strains [33]. Further investigations are underway to elucidate the reason behind the differing effects of divalent cations on antimicrobial efficacy against different bacterial species.

To explore the effect of divalent cations on the antimicrobial mechanism of SNAPPs, an OM permeability assay using a hydrophobic 1-N-phenylnaphthylamine (NPN) fluorescent probe was conducted, as OM disruption has been shown in Chapter 2 to be crucial to the antimicrobial mechanism of SNAPPs [24]. NPN emits weak fluorescence in an aqueous environment and only fluoresces strongly in a hydrophobic environment such as in the bacterial membrane [34]. There are no chemical structural changes involved when the dye is switched on or off. As NPN, like most hydrophobic substances, is normally excluded by intact OMs, an increase in fluorescence intensity when incubated with Gram-negative bacterial cells would indicate disruption to the OM. Bacterial cells (either *E. coli* or *A. baumannii*) were added to varying concentrations of SNAPP S16 in the presence of NPN, and the fluorescence intensity (expressed in terms of relative fluorescence units, RFU) was measured as a function of time (Figure 4.2). The medium used was either SBF or mSBF. In all cases, regardless of the bacterial species and the type of medium used, the increase in NPN fluorescence intensity was found to reach a maximum for all polymer concentrations within 90 min.
Figure 4.2. 1-N-phenylnaphthylamine (NPN) uptake of *E. coli* and *A. baumannii* induced by SNAPPS16. 

(a) and (b) cells were added to NPN in the presence of increasing concentrations of S16 (0.05-2.92 µM). NPN fluorescence was monitored at an excitation wavelength of 350 nm and an emission wavelength of 420 nm starting at $t = 5$ min after the addition of bacterial cells and continued until a maximum is reached (if not at $t = 5$ min). NPN fluorescence was expressed in terms of relative
fluorescence units (RFU). Controls whereby no S16 was added (0 µM) were included. The assays were conducted in either SBF (black) or mSBF (red). For E. coli, an additional assay was performed in SBF with 1.5 mg/mL of EDTA (blue).

When E. coli cells were treated with S16 in mSBF, NPN was taken up in a dose-dependent manner and the RFU recorded at each concentration reached its maximum 5 min after the addition of bacterial cells (Figure 4.2a). The increase in RFU with S16 concentration reached a plateau at about 35000 RFU at a concentration of 0.73 µM (Figure 4.2a). As the MIC of S16 in mSBF was found to be 0.22 µM, it was postulated that the extent of OM damage induced at this concentration (correlating to an RFU of ca. 27500) is the OM disruption threshold needed to result in bacterial growth inhibition. The further increase in RFU above the threshold at supra-MIC dosages could be attributed to more drastic membrane lysis.

In SBF, only a slight increase in NPN uptake with S16 concentration was observed (RFU ≤ 5000) 5 min after the addition of E. coli cells (Figure 4.2a). The RFUs across all S16 concentrations continued to increase with time until 90 min post-cell addition. However, it was noted that at 0.22 µM (MIC of S16 in mSBF), the RFU recorded in SBF was still very low (ca. 5000) even after 90 min, which indicated that the extent of OM disruption at this concentration was unlikely to be significant in the complete medium. A relatively high RFU of 25000 (close to the membrane disruption threshold of 27500) was only achieved at the highest concentration tested of 2.92 µM after 90 min, which closely correlated with the MIC (Table 4.2).

Similar to the observations made for E. coli, the NPN uptake of A. baumannii proceeded in a dose-dependent fashion (Figure 4.2b). When mSBF was used as the medium, prominent NPN uptake was observed within a short time (i.e., 5 min), even at a low dosage of 0.05 µM (corresponding to an RFU of ca. 48000). On the other hand, in SBF, NPN uptake across all SNAPP concentrations was low initially, but increased with time and became constant at 25 min. At 25 min, the RFUs recorded using SBF as the medium were close to those measured in mSBF at the corresponding concentrations, albeit slightly lower. It is worthwhile noting that at concentrations that corresponded to the MICs of S16 in mSBF (0.13 µM) and SBF (0.17 µM), RFUs that were above 40000 were achieved. Collectively, these results, together with those obtained when tested against E. coli, suggested that an OM disruption threshold needs to be reached for SNAPPs to induce bacterial cell death. The mode of action of AMPs is commonly thought to proceed through CM disruption [5,35,36], and
studies on the interactions between AMPs and the OM are limited [37]. This study highlights the importance of OM disruption for efficacy against Gram-negative bacteria and hence, this should be considered when investigating the antibacterial mechanism of other AMPs.

Subsequently, in order to better understand the effects of different media on polymer-OM interactions, the initial increase in RFU as a function of S16 concentration (slope of curve before a plateau was reached) was compared across the different media and time points (Table 4.3). Against E. coli, it was noted that the slope obtained at 5 min when mSBF was used was ca. 150000 RFU/µM. However, when the NPN assay was conducted in SBF, the slope was smaller and remained relatively constant (43422-57017 RFU/µM) even when the incubation time was increased from 5 to 90 min. In the case of A. baumannii, while the slope obtained after a 5-min incubation in SBF was significantly (ca. 8 times) lower than that in mSBF, it increased by close to 4-fold when the incubation time was extended to 25 min.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Incubation time (min)</th>
<th>Slope (RFU/µM)</th>
<th>E. coli</th>
<th>A. baumannii</th>
</tr>
</thead>
<tbody>
<tr>
<td>mSBF</td>
<td>5</td>
<td>150803</td>
<td>691755</td>
<td></td>
</tr>
<tr>
<td>SBF</td>
<td>5</td>
<td>48344</td>
<td>85629</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>57017</td>
<td>327942</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>43422</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SBF + 1.5 mg/mL EDTA</td>
<td>5</td>
<td>131362</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Based on these results (Figure 4.2a and Table 4.3), it was theorized that the divalent cations (from the MgCl2·6H2O and CaCl2 salts in SBF) drastically reduced the interaction between SNAPP S16 and the OM of E. coli. The decrease in interaction was independent of the polymer-bacteria incubation time. The extent of OM disruption was consequently antagonized, as a higher concentration of S16 was needed to cause microbial growth inhibition. It was further hypothesized that this postulation could be applied to account for the
reduction in activity of SNAPPs observed against *P. aeruginosa* and *K. pneumoniae* in SBF. On the other hand, when tested against *A. baumannii*, it was postulated that the presence of divalent cations resulted in an initial lag time where the polymer-bacteria interaction was prevented. When incubation was prolonged beyond the lag time, OM permeabilization was initiated to effect sufficient damage to the OM, resulting in an MIC that was similar to that when the divalent cations were absent. It was also observed that the slopes obtained when tested against *A. baumannii* were substantially higher (*e.g.*, 4.5 times higher in mSBF) than those obtained against *E. coli* regardless of the medium used. This indicated the enhanced ability of SNAPPs to disrupt the OMs of *A. baumannii* compared to those of other bacterial species.

*A. baumannii* is an opportunistic pathogen that is responsible for a significant number of hospital-acquired infections and has become extensively resistant to numerous antibiotics [38]. SNAPP S16 has been demonstrated to possess excellent *in vivo* efficacy against wild-type and colistin-multidrug-resistant (CMDR) *A. baumannii* species. The results herein substantiated the potential of SNAPPs as effective antimicrobial agents against *A. baumannii* infections as their potency against this species was retained at physiologically relevant salt concentrations. Despite the reduction in efficacy against the other bacterial species tested (*i.e.*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*), SNAPPs were still able to maintain good MIC values within the range of 0.59 to 4.68 µM, which are superior (*e.g.*, >20 times more effective against *E. coli*) to the MICs of several AMPs, such as LL-37, indolicidin, defensin, and magainin I [39].

Next, a CM disruption assay was conducted to validate the species-dependent effects of divalent cations on the antimicrobial activity of SNAPPs. Based on earlier observations (Figure 4.2), the SNAPP-cell interactions occurred within 90 min of incubation, regardless of the medium used. For the CM disruption assay, SNAPP S16 was incubated with either *E. coli* or *A. baumannii* cells for 90 min, and then a mixture of SYTO® 9 green fluorescent nucleic acid stain and propidium iodide (PI), a red fluorescent nucleic acid stain, was added. The SYTO® 9 dye is membrane-permeable and stains the nucleic acid of all bacterial cells, while the membrane-impermeant PI only labels bacteria with damaged IMs. In this study, as all bacterial species investigated are Gram-negative, a bacterial cell that is ‘PI-positive’ would possess damaged OM and CM (see Figure 4.3a - illustration). The untreated *E. coli* and *A. baumannii* cells were found to have 1.9% and 14.7% of PI-positive cells, respectively (Figure 4.3a and b). The cells were then incubated with S16 at 0.09 µM (a sub-inhibitory
concentration that approximates one-half of the MIC of S16 in MEM) for 90 min in different media (either MEM, SBF or mSBF) and the resulting percentage of PI-positive cells was compared (Figure 4.3a and b). A sub-inhibitory concentration was used, instead of the lethal concentration, to prevent the occurrence of cell fragmentation or lysis which would reduce the size of the bacterial cell population detectable by the flow cytometer. When tested against E. coli in SBF, only 10.1% of cells were PI-positive compared to 55.8% in MEM. However, in mSBF which has no Mg\(^{2+}\) and Ca\(^{2+}\) ions, the proportion of membrane-disrupted cells increased to 63.7% (comparable to that in MEM). On the other hand, against A. baumannii, the percentage of PI-positive cells in MEM and SBF were similar (96.4% and 93.7%, respectively). The results validated the postulation that the presence of Mg\(^{2+}\) and Ca\(^{2+}\) ions at biologically-relevant concentrations attenuated the extent of membrane disruption in E. coli caused by S16, whereas against A. baumannii the membrane disrupting ability of SNAPP S16 was not affected.

![Figure 4.3](image_url)

**Figure 4.3. Uptake of propidium iodide (PI) by E. coli and A. baumannii cells before and after treatment with SNAPP S16.** a-c, E. coli (a, c) or A. baumannii (b) cells were incubated with S16 for 90 min at a sub-MIC of 0.09 µM. After incubation the cells were stained with SYTO\(^{9}\) and PI nucleic acid dyes prior to analysis. On the two-parameter dot
plots obtained by flow cytometry, the x-axis represents fluorescent channel 1 (FL1), which measures the fluorescent emission of SYTO® 9. The y-axis represents fluorescent channel 3 (FL3), which measures the fluorescent emission of PI. Cells were determined to be ‘PI-positive’ if fluorescence emitted is captured by FL3. Controls whereby no S16 was added (0 µM) were included. For E. coli, the assays were conducted in MEM (a), SBF (a), mSBF (a), 10% serum in MEM (c), or SBF with 1.5 mg/mL EDTA (c). For A. baumannii, assays were conducted in either MEM or SBF (b).

4.3.2 Effect of Chelating Agent Addition

It was postulated that the addition of a chelating agent such as ethylenediaminetetraacetic acid (EDTA) disodium salt which chelates Ca$^{2+}$ and Mg$^{2+}$ ions could attenuate the antagonistic effect of the divalent cations on antimicrobial activity. To validate this, E. coli cells were treated with SNAPP S16 and varying concentrations of EDTA disodium salt, and a reduction in MIC of S16 with increasing concentrations of EDTA disodium salt was observed (Figure 4.4). The lowest MIC of S16 achieved was 0.42 ± 0.02 µM at an EDTA disodium salt concentration of 1.5 mg/mL. The chelating agent was found to restore the OM disrupting ability of SNAPP S16 to the same rate and extent as in the modified SBF without any divalent cations (i.e., mSBF) (Figure 4.2a and Table 4.3). The CM permeabilization ability of S16 was also restored (Figure 4.3c). Further increase in EDTA disodium salt concentration did not lead to further reduction in MIC. It should be noted that the addition of EDTA at all concentrations tested did not affect the bacterial cell viability. Although there are safety concerns regarding the use of EDTA for in vivo applications, strategies such as the use of more biocompatible chelators (e.g., citric acid), tethering of the chelator to the SNAPP through chemical conjugation, on-demand release of the chelator at the infection site, or any combination thereof, could possibly improve the therapeutic utility of such SNAPP-chelator drug combination. Further, this formed a proof-of-concept study to demonstrate that challenges posed by inhibitory substances present in vivo such as divalent cations could potentially be circumvented.
Figure 4.4. Effect of EDTA on antimicrobial activity of SNAPP S16 against *E. coli*. *E. coli* cells were treated with varying concentrations of S16 (0.05-4 µM) and EDTA disodium salt (0.5-2 mg/mL), and the MICs of S16 were evaluated. All data are expressed as mean ± standard deviation as indicated by the error bars (n = 4).

4.3.3 Antimicrobial Studies in Serum

To facilitate the evaluation of SNAPPs S16 and S32 for possible therapeutic utility, an *ex vivo* assay was developed to assess their antimicrobial efficacies in animal serum (*i.e.*, fetal bovine serum (FBS); diluted to 10% and 50% in MEM). Serum is a cell-free biomatrix isolated from the coagulation of blood and plasma components, with associated activation of proteases and other factors [3]. Besides salts which have been shown to have an inhibitory effect on antimicrobial activity, blood serum contains serum albumin and a range of other proteins such as transferrin and lactoferrin which could collectively form a protein corona around antimicrobials and decrease their therapeutic activity by reducing the free fraction of drug or nanoparticle available for bacteria association and/or killing [12,13,40].

When 10% FBS in MEM was used, the MICs of SNAPPs S16 and S32 increased significantly by *ca.* 3 to 17 times depending on the peptide polymer and bacterial species tested against (Table 4.2). The activity of SNAPP S32 against *K. pneumoniae* was reduced by almost 10 times, while S16 became non-active against *K. pneumoniae* within the range of concentrations tested (≤6 µM). When tested against *A. baumannii*, a relatively weaker effect
(ca. 3 to 5-fold reduction in activity) was observed for both S16 and S32. These observations substantiated the potential of SNAPPs as effective antimicrobials against A. baumannii even under physiologically-relevant conditions. The reduction in activity (albeit to different extents depending on serum concentration and bacterial species tested against) may be attributed to weaker membrane disruption activities in the presence of serum. The extent of membrane disruption caused by SNAPP S16 against E. coli, as indicated by flow cytometry, dropped from 55.8% in MEM to 10.2% in 10% serum (Figure 4.3c). The inhibitory activities of SNAPPs (against E. coli and P. aeruginosa, as shown in Table 4.2) were further antagonized when the serum concentration was increased to 50%, close to the serum content in blood. It should be noted that as a result of the lack of activity even in 10% serum, S16 and S32 was not tested against K. pneumoniae in 50% serum. Antimicrobial tests against A. baumannii in 50% serum were not possible as bacteria viability in the untreated control was affected significantly, possibly due to the intrinsic antimicrobial activity of serum [3]. The reason as to why a high serum concentration was detrimental to the growth of the A. baumannii strain used herein and not to that of the other bacterial species tested requires further investigations.

Serum consists of salts and proteins, of which serum albumin is a major component of the latter. In Chapter 2, the non-specific binding of SNAPPs with medium contents in protein-rich media (MHB and FBS) was demonstrated through dynamic light scattering (DLS) analysis [24]. To determine if the reduction in activity in serum was due to protein adsorption, inhibition by divalent cations, or both, the components present in serum were isolated and the effects of each component on antimicrobial activity were investigated. The MICs of S16 and S32 against E. coli in 10% serum were first compared to the MICs in (i) 10% SBF (diluted in MEM) to simulate the ionic composition in 10% serum, and (ii) in 0.22 mg/mL bovine serum albumin (BSA) (dissolved in MEM) which approximates the amount of albumin present in 10% serum (Figure 4.5a). For both S16 and S32, no significant difference in the MICs obtained in either 10% SBF or 0.22 mg/mL BSA in MEM was noted. Further, the MICs in these media were higher than the MICs in MEM, but still lower than the MICs obtained in 10% serum. This suggested that both salts and BSA, when present alone, affected the efficacy of SNAPPs to a similar extent. When both were present together in the test medium such as in serum, an antagonistic effect that caused further reduction in potency was resulted. It was also likely that other serum proteins besides albumin could contribute to the lowering of antimicrobial efficacy. Further, it was noted that S32 was less affected by the protein or salt content found
in 10% serum as compared to S16, as the MIC of S32 was reduced to a lesser extent (2-fold) compared to S16 (4-fold) when either protein or salt was removed from 10% serum. This suggested a possible relationship between peptide polymer valency and the salt and protein resistance ability of the polymers, which could be explored in future studies. It should also be noted that polymer-protein interactions would be influenced by other factors, such as protein and polymer charges, hydrophilicity and hydrophobic interactions [41].

![Figure 4.5. Antimicrobial activity of SNAPPs S16 and S32 against E. coli in different test media.](image)

A similar investigation was conducted at a higher serum concentration of 50%, i.e. by comparing the MIC in 50% serum to those in 50% SBF in MEM and in 1.1 mg/mL BSA (which is the albumin concentration in 50% serum) in MEM (Figure 4.5b). Similarly, SNAPPs produced a weaker growth inhibitory effect against E. coli in either 50% SBF or 1.1 mg/mL BSA in MEM, compared to the case when 50% serum was used. Both salts and serum proteins were found to have an antagonistic effect on antimicrobial activity when present together in 50% serum. Interestingly, it was noted that the MICs of S16 and S32 were ca. 2 times higher in 50% SBF than in 1.1 mg/mL BSA in MEM. This suggested that at higher salt concentrations such as that found in 50% serum, the presence of salts seemed to inhibit antimicrobial activity to a greater extent compared to BSA.
4.3.4 Effects of Different Biological Environments on Polymer-Bacteria Interactions

The effects of different test media on SNAPP-bacterial cell interactions are illustrated in Figure 4.6. Compared to the antimicrobial mechanism of SNAPPs in MHB or MEM as reported in Chapter 2 (Figure 4.6a) [24], it was postulated that the presence of divalent cations (Ca$^{2+}$ and Mg$^{2+}$) at physiological concentrations stabilizes the LPS layer and thus hinders the ability of SNAPPs to bind, and destabilize or fragment the OMs (Figure 4.6b). The addition of a chelating agent such as EDTA disodium salt which chelates Ca$^{2+}$ and Mg$^{2+}$ could attenuate the antagonistic effect of the divalent cations on antimicrobial activity (Figure 4.6c). In the presence of blood serum which contains proteins and salts, protein binding to SNAPPs results in further reduction in antimicrobial efficacy (Figure 4.6d).

**Figure 4.6. Proposed effects of different medium environments on SNAPP S16-bacterial OM interaction.** In MEM, S16 was thought to interact with the LPS layer on the OM via electrostatic interactions and cause OM destabilization and fragmentation (a). Divalent cations, when present in the medium, stabilize the LPS layer and inhibit the ability of SNAPPs to disrupt the OM (b). The addition of a chelating agent restores the membrane disrupting ability of SNAPPs (c). In the presence of serum proteins, the formation of SNAPP-protein aggregates reduces SNAPP-membrane interaction (d).
4.4 Chapter Summary

In conclusion, a comprehensive study is presented on the effects of different medium environments on the antimicrobial activity of the previously reported SNAPPs (in the form of star-shaped peptide polymers). The efficacy of SNAPPs under *in vivo* mimicking environments was evaluated and supplemented with membrane permeability assays and flow cytometric analysis. When tested in simulated body fluid (SBF) which mimics the ionic composition *in vivo*, the inhibitory effect caused by divalent cations affected the activity of SNAPPs against *E. coli*, *K. pneumoniae* and *P. aeruginosa* with a substantial increase in their MICs. Using *E. coli* as a representative bacterial species, it was demonstrated that divalent cations (especially Ca$^{2+}$ ions) reduced the ability of SNAPPs to permeabilize the outer membrane (OM) as well as cause cytoplasmic membrane (CM) disruption. When tested against *A. baumannii* in the presence of divalent cations, the rate at which SNAPPs disrupted the OM was reduced initially but the disruption ability was recovered within reasonable timescales and the MIC was not affected. In blood serum, besides the presence of divalent cations, the binding of serum proteins to SNAPPs reduced their ability to act on bacterial membranes. The presence of both salts and proteins resulted in an antagonistic effect on the antimicrobial efficacy of SNAPPs, leading to a significant decrease in activity compared to the case when either salts or proteins were present. Overall, this study demonstrated the need to perform antimicrobial susceptibility assays under conditions that are relevant to their potential therapeutic applications *in vivo*. The results presented herein provided an understanding of the interaction of SNAPPs with inhibitory compounds and biomolecules present *in vivo*, which could aid in the development of optimized SNAPPs with high protein and salt resistance, and hence the ability to retain clinical effectiveness under physiological conditions. It is also noteworthy that the study highlighted the potential of SNAPPs as a novel antimicrobial agent against *A. baumannii* infections, as potency was retained in high salt concentrations and the MIC was not substantially affected in the presence of serum proteins.
4.5 References


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CHAPTER 5

Star Peptide Polymers as an Antibiotic Adjuvant to Restore Antibiotic Sensitivity to Colistin-Multidrug Resistant Bacteria

5.1 Chapter Perspective

The rise and prevalence of multidrug-resistant Gram-negative bacteria has resulted in an urgent need for innovative approaches to treat life-threatening infections with these bacteria. Synergistic therapy has been proposed as a practical solution where multiple antibiotics are administered in combination to achieve substantially greater efficacy than when administered individually. Studies reported in this area are mostly limited to antibiotic pairings, and specific combinations are often needed to combat a selective range of bacterial species. In this chapter, ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs) were shown to act as an effective adjuvant to different classes of conventional antibiotics in combating Gram-negative pathogens, including colistin and multidrug-resistant (CMDR) species. Synergistic interactions were demonstrated between a model SNAPP and ampicillin, imipenem, doxycycline, gentamicin, or silver ions. It was also shown that the effective antibiotic dose against antibiotic-resistant bacteria could be decreased by 3.7 to 16-fold from the original lethal dose through synergistic interactions with SNAPPs. Further, it was demonstrated that the combination treatment approach using SNAPPs is able to attenuate toxicity. Overall, this study demonstrated the potential of synergistic combinations of SNAPPs with conventional antibiotics where the SNAPP not only has antimicrobial activity in its own right, but also acts as an adjuvant to potentiate and restore the efficacy of conventional antibiotics against drug-resistant bacterial infections.
5.2 Introduction

The prevalence of antimicrobial resistance and the fact that few new antibiotics are in development have given rise to an urgent need for the development of novel yet practical approaches to overcome multidrug-resistance (MDR) bacterial infections. One potential solution is through synergistic therapy where multiple antimicrobial compounds are co-administered during a treatment regimen with the aim of achieving an effect greater than the sum of the individual treatments [1]. Combination therapy is being widely used in the treatment of many health conditions such as cancer, tuberculosis and viral infections [2-4], and has recently been regarded as a promising and cost-effective solution for bacterial infections to overcome the inadequacies of antibiotic monotherapy [4]. Through the combination of antimicrobial drugs that interact with different and multiple bacterial targets, synergistic therapy could have profound implications in (1) reducing the likelihood of resistance acquisition [3,5,6], (2) re-sensitizing MDR bacteria to antibiotics that are otherwise ineffective when administered alone [4,7], and (3) mitigating toxic side-effects to the body as similar levels of antimicrobial efficacy could be attained with lower drug concentrations [5].

While synergistic antibiotic-antibiotic combinations are the most commonly reported [3,4,8], most antibiotic cocktails tend to be only effective against selective bacterial species and such combinations have also been reported to be associated with greater resistance due to increased selective pressure [3,9,10]. Therefore, recent studies have been focusing on the combination of antibiotics with antimicrobials that are known to impede resistance development, such as membrane-active antimicrobial peptides (AMPs) [11-21] and polymers [5,22-24]. However, such approaches are not without their drawbacks, as membrane-active antimicrobials typically have non-specific activity and hence suffer from high dose-limiting toxicity to host cells [2,5]. Further, studies done on synergistic antibiotic-polymer combinations are scarce and efficacy has only been demonstrated against a limited range of bacterial species. In the fight against MDR Gram-negative infections, synergistic therapy is often a combination of colistin - a nephrotoxic drug regarded as the ‘last line of defense’ [25] - with another antibiotic [4]. With increasing reports on the rise of colistin-resistant and MDR (CMDR) bacteria, there is an urgent need for novel therapies. One approach is the concept of an antibiotic adjuvant, whereby a conventional antibiotic’s efficacy is potentiated or restored by co-administration of either a non-antibiotic molecule or another antimicrobial agent, resulting in enhanced or synergistic activity, respectively [26,27].
SNAPPs have been demonstrated to possess broad spectrum efficacy against wild-type and CMDR Gram-negative pathogens [28]. They were also found to exhibit low toxicity towards mammalian cells (unlike AMPs) and not induce resistance due to their unique, multi-modal mechanism of action. Based on these attributes, it was hypothesized that SNAPPs may be promising adjuvants for conventional antibiotics via combination therapy to potentiate activity against MDR Gram-negative bacteria. In this chapter, a model SNAPP was shown to be able to synergize with different classes of conventional antibiotics against a range of Gram-negative bacteria, including opportunistic ESKAPE pathogens, which are named for their ability to ‘escape’ antibiotic action [29,30], and CMDR clinical isolates. The synergistic interactions resulted in effective polymer and antibiotic dose reductions as well as the resensitization of drug-resistant bacteria to certain antibiotics. The use of SNAPPs as an antibiotic adjuvant was also demonstrated to be capable of overcoming toxicity issues associated with antibiotic monotherapy.

5.3 Results and Discussion

5.3.1 In Vitro Screening for SNAPP-Antibiotic Synergism

In this chapter, all experiments were conducted using a model SNAPP in the form of a nano-sized, 16-arm star-shaped peptide polymer, S16, comprising lysine and valine amino acid residues (Figure 5.1). The synthesis and characterization of S16 are as previously described in Chapter 2 [28]. The findings in Chapter 2 suggested that the mechanism of action of S16 involves outer membrane (OM) destabilization, unregulated ion movement across the cytoplasmic membrane (CM), and induction of the apoptotic-like death pathway, ultimately resulting in cell lysis [28]. As the antimicrobial action is multi-modal where SNAPPs are able to act on multiple cellular targets that are different from antibiotic targets (a prerequisite of an antibiotic adjuvant [26,27]), it was hypothesized that S16 could potentially interact synergistically with different classes of antibiotics, either by combined multi-modal antimicrobial activities and/or by potentiating or restoring the activity of the conventional antibiotic by acting as an adjuvant.
Figure 5.1. Chemical structure of SNAPP S16.

As an initial step, S16 was screened in combination with a model β-lactam (ampicillin), an aminoglycoside (gentamicin), and silver (in the form of dissolved ions, Ag⁺, from AgNO₃) for synergy against wild-type strains of Escherichia coli, Klebsiella pneumoniae, P. aeruginosa, and A. baumannii, all of which are clinically-relevant Gram-negative pathogens. The antibacterial activities of each compound are reported as minimum bactericidal concentrations (MBCs) in Appendix III, Supplementary Table 5.1. MBC determination was selected as the method of choice to evaluate antimicrobial efficacies as it quantifies the concentration needed to cause bacterial cell death and hence is a more stringent approach than the minimum inhibitory concentration (MIC) analysis which measures growth inhibition [31]. To determine the extent of synergy between a SNAPP S16-antibiotic pair, bacterial cells were treated with sub-lethal concentrations of the compounds (i.e., MBC50 which is one-half of the MBC), either individually or in combination based on the method reported by Collins and co-workers [7]. The change in the log of colony-forming unit (CFU) per mL, log(CFU/mL), compared to the untreated control at time zero was monitored after 1.5 (Appendix III, Supplementary Figure 5.1) and 3 h (Figure 5.2).
Figure 5.2. Screening analysis for synergistic S16-antibiotic pairs. a–f, Log change in colony-forming units (CFU)/mL of E. coli (a), K. pneumoniae (b), P. aeruginosa (c), CMDR P. aeruginosa (d), A. baumannii (e) and CMDR A. baumannii (f) from time zero after treatment for 3 h with S16, a range of antibiotics, and combinations of S16 with the respective antibiotics, with all compounds at their MBC50. All data are expressed as mean ± standard deviation as indicated by the error bars (n = 3). ***P < 0.001, **P < 0.05, Student’s t test. Amp = ampicillin, Gen = gentamicin, Dox = doxycycline, Tob = tobramycin, Imi = imipenem.

At t = 3 h, significantly enhanced antimicrobial activity (as indicated by increased bacterial cell death) was observed against the four bacterial species tested when S16 was co-administered with either ampicillin or AgNO₃ (P<0.05), compared to when the compounds were administered alone (Figure 5.2a–c, e). When S16 and gentamicin were co-administered, enhanced efficacy was only observed against E. coli and K. pneumoniae (P<0.001) (Figure 5.2a–b). The effects of co-administration were further investigated by combining S16 with other antibiotics, such as doxycycline (which is used in the treatment of respiratory infections...
caused by *P. aeruginosa* [32, 33]), imipenem (a common treatment option for serious infections caused by *A. baumannii* [34]), and tobramycin (an aminoglycoside commonly used to treat Gram-negative infections such as those caused by *P. aeruginosa* [35] and *A. baumannii* [34]). Increased efficacy was observed for S16-doxycycline and S16-imipenem combinations against *P. aeruginosa* and *A. baumannii*, respectively (*P*<0.001) (Figure 5.2c and e). Interestingly, antagonism between S16 and tobramycin was indicated when tested against *P. aeruginosa* and *A. baumannii*, as demonstrated by the reduction in bactericidal efficacy compared to when the antibiotic was administered alone (Figure 5.2c and e).

For *P. aeruginosa* and *A. baumannii*, CMDR clinical isolates were also tested in addition to the wild-type strains (Figure 5.2d and f; refer to Appendix I, Supplementary Tables 2.4 and 2.5 for antibiograms). Enhanced antimicrobial activity was demonstrated by the co-administration of S16 with either AgNO$_3$ or doxycycline against CMDR *P. aeruginosa* (*P*<0.05), and S16 with ampicillin, AgNO$_3$ or imipenem against CMDR *A. baumannii* (*P*<0.001). Similar to that observed for wild-type *P. aeruginosa* and *A. baumannii*, the combination of S16 and tobramycin against the CMDR isolates was antagonistic. The pairing of S16-ampicillin against CMDR *P. aeruginosa* was deemed redundant and hence not investigated as the bacterial strain used is already highly susceptible to ampicillin (MBC<1.4 µM). Further, S16-gentamicin was excluded from further investigations with the CMDR isolates as this pairing did not result in any significant change in activity against the wild types compared to the case when the compounds were administered alone.

It is noteworthy that at a shorter incubation time of 1.5 h, the results obtained were similar to those obtained at *t* = 3 h, albeit with less pronounced effects for the cases where S16-antibiotic combination therapy resulted in an increase in efficacy (Appendix III, Supplementary Figure 5.1). An exception was noted where the co-administration of S16 with gentamicin resulted in enhancement in efficacy against *P. aeruginosa* at *t* = 1.5 h (*P*<0.05), but not at *t* = 3 h. This could be explained by the growth of the remaining viable cells when incubated beyond 1.5 h with S16-gentamicin, resulting in an increase in cell counts at *t* = 3 h compared to that at 1.5 h.

5.3.2 Evaluation of the Degree of Synergy

Following the observations made from the screening analysis (Figure 5.2), the Bliss Independence and Highest Single Agent (HSA) models (see Chapter 7.8, “Quantifying synergy using the Bliss Independence and Highest Single Agent (HSA) models” for details)
were used to evaluate the degree of synergy based on the 3 h time point, which would quantitatively determine if the antimicrobial effect between two compounds was synergistic. The results are shown in Table 5.1. The two models, while independent of each other, were found to produce identical evaluations of synergism, except in one case where the Bliss Independence model computed functional synergy between S16 and gentamicin against A. baumannii, while the HSA model indicated otherwise. As the HSA model has been reported to be a more stringent method to evaluate drug synergism [36], the aforementioned pair was determined to be non-synergistic against A. baumannii. Based on the models, S16 was found to synergize with Ag⁺ (from AgNO₃) against all six bacterial species tested. The combination of S16 with either doxycycline or imipenem was also found to be synergistic against all bacterial species tested (wild-type and CMDR P. aeruginosa for S16-doxycycline; wild-type and CMDR A. baumannii for S16-imipenem). The antimicrobial effects resulting from the S16-ampicillin combination were found to be synergistic against K. pneumoniae, P. aeruginosa, and CMDR A. baumannii. While Figure 5.2 indicated statistically significant enhancement of activity (P<0.05) against E. coli and A. baumannii following co-administration of S16 and ampicillin, the antimicrobial effects were not sufficient to satisfy the criteria for synergism based on either the Bliss Independence or HSA models. In these cases, the interactions between the two compounds against the aforementioned bacterial species were deemed ‘additive’ (i.e., an effect equal to the sum of the treatments) at most. As expected, the S16-tobramycin pairing was found to be non-synergistic against the bacterial species tested.
Table 5.1. Evaluation of the Absence/Presence of Synergy between S16 and a Range of Antibiotics Using the Bliss Independence and Highest Single Agent (HSA) models

<table>
<thead>
<tr>
<th>MBC in combination (µM)</th>
<th>Synergistic? (Yes/No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotics</td>
<td>S16</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
</tr>
<tr>
<td>Amp</td>
<td>5.7</td>
</tr>
<tr>
<td>Gen</td>
<td>2.2</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>K. pneumoniae</strong></td>
<td></td>
</tr>
<tr>
<td>Amp</td>
<td>327.6</td>
</tr>
<tr>
<td>Gen</td>
<td>1.1</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>5.3</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
</tr>
<tr>
<td>Amp</td>
<td>2930.7</td>
</tr>
<tr>
<td>Gen</td>
<td>1.2</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>0.5</td>
</tr>
<tr>
<td>Dox</td>
<td>25.3</td>
</tr>
<tr>
<td>Tob</td>
<td>1.9</td>
</tr>
<tr>
<td><strong>CMDR P. aeruginosa</strong></td>
<td></td>
</tr>
<tr>
<td>Ag⁺</td>
<td>11.8</td>
</tr>
<tr>
<td>Dox</td>
<td>295.3</td>
</tr>
<tr>
<td>Tob</td>
<td>119.7</td>
</tr>
<tr>
<td><strong>A. baumannii</strong></td>
<td></td>
</tr>
<tr>
<td>Amp</td>
<td>1465.4</td>
</tr>
<tr>
<td>Gen</td>
<td>6.0</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>4.1</td>
</tr>
<tr>
<td>Imi</td>
<td>19.0</td>
</tr>
<tr>
<td>Tob</td>
<td>7.6</td>
</tr>
<tr>
<td><strong>CMDR A.baumannii</strong></td>
<td></td>
</tr>
<tr>
<td>Amp</td>
<td>2930.7</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>2.9</td>
</tr>
<tr>
<td>Imi</td>
<td>177.4</td>
</tr>
<tr>
<td>Tob</td>
<td>2190.3</td>
</tr>
</tbody>
</table>
Out of the 23 different \textbf{S16}-antibiotic-bacteria combinations investigated, 15 were found to exhibit synergism between \textbf{S16} and the antibiotic used. The adjuvanticity of SNAPP \textbf{S16} may be attributed to its ability to disrupt the bacterial membrane, as shown by the dose-dependent increase in the uptake of the membrane-impermeable propidium iodide (PI) dye (Appendix III, Supplementary Figure 5.2). Furthermore, the treatment of bacterial cells with \textbf{S16} also led to membrane potential alterations, causing the formation of mixed hyperpolarized and depolarized bacterial cell populations (Appendix III, Supplementary Figure 5.2). Bacterial membrane disruption and membrane potential dissipation have been reported to result in the abrogation of efflux pumps and the enhanced uptake of antibiotics [26,37]. As the active efflux of antibiotics is one of the major mechanisms for antibiotic resistance [27], it was proposed that the membrane perturbations caused by SNAPPs may account for their adjuvant role in potentiating antibiotic activity against CMDR bacterial species. Further, it was postulated that the excellent synergism between \textbf{S16} and Ag\textsuperscript{+} may be explained by the similarities in the antimicrobial mechanism of both compounds, where treatment of Gram-negative bacteria with either SNAPPs or Ag\textsuperscript{+} resulted in increased reactive oxygen species (ROS) production and membrane permeability, as reported in Chapter 2 [7,28]. This postulation is consistent with previous studies, where high functional synergy between two antimicrobial compounds was attributed to mechanistic analogy between the compounds [2].

On the other hand, the general lack of synergism between \textbf{S16} and the aminoglycoside antibiotics tested, as exemplified by the indifference shown by \textbf{S16}-gentamicin towards half of the bacterial species tested and the antagonism between \textbf{S16} and tobramycin against all four bacterial species tested, may be attributed to the competition for cationic binding sites on the lipopolysaccharide (LPS), which is found on the OM of Gram-negative bacteria. As binding with the LPS layer is the first step in the mode of action of both \textbf{S16} and aminoglycosides in general [1,28], the reduction in available binding sites could lead to a reduction in efficacy for each compound and this may result in the lack of synergy or, in some cases, antagonism as observed. This phenomenon was also reported by Khalil \textit{et al.} to account for the antagonism between polyethyleneimine, a highly cationic antimicrobial polymer, and aminoglycosides such as tobramycin [22]. It was noted that the degree of synergy for the pairing between \textbf{S16} and ampicillin or gentamicin seemed to vary depending on which bacterial species was tested. This could possibly be related to the differences in composition and properties of the OMs of Gram-negative bacteria [38]; therefore, further
investigation is needed to elucidate the species-dependent effects observed for such drug combinations.

5.3.3 Effects of Antibiotic Concentration on Synergism

Next, five \textit{S16}-antibiotic-bacteria combinations, where the bacterial species was found to be resistant to the respective antibiotic, were selected to investigate the effects of antibiotic concentration on \textit{S16}-drug synergism. The combinations chosen were \textit{S16}-ampicillin against wild-type ampicillin-resistant \textit{P. aeruginosa}, \textit{S16}-Ag\textsuperscript{+} against CMDR isolates of \textit{P. aeruginosa} and \textit{A. baumannii}, \textit{S16}-doxycycline against CMDR \textit{P. aeruginosa}, and \textit{S16}-imipenem against CMDR \textit{A. baumannii}. In each case, the \textit{S16} concentration was fixed at its MBC50. Based on Figure 5.3, increased cell death ($P<0.05$) following combination treatments for 3 h was observed for all cases even when the antibiotic concentration was reduced below the MBC50 of the antibiotic. For treatments against wild-type and CMDR \textit{P. aeruginosa} in particular, the antibiotic concentrations could be reduced by 8 to 32-fold from the MBC50 of the antibiotic when administered alone (Figure 5.3a-c). However, it should be noted that a decreasing trend in the extent of efficacy enhancement was observed as the antibiotic concentration used was lowered. The experiments were also conducted in different variations, where either the \textit{S16} concentration was decreased to its MBC25 or the incubation time was shortened to 1.5 h (Appendix III, Supplementary Figures 5.3-5.5). Generally, enhanced antimicrobial activity ($P<0.05$) was demonstrated in most cases following reductions in antibiotic concentration, although the effects were less prominent.
CHAPTER 5

Figure 5.3. a-e, Log change in CFU/mL of various bacterial species from time zero after treatment for 3 h with S16 at its MBC50 and a range of antibiotics at the indicated concentrations. The synergistic pairs investigated are S16-ampicillin (Amp) against P. aeruginosa (a), S16-AgNO₃ (Ag⁺) against CMDR P. aeruginosa (b), S16-doxycycline (Dox) against CMDR P. aeruginosa (c), S16-AgNO₃ (Ag⁺) against CMDR A. baumannii (d), and S16-imipenem (Imi) against CMDR A. baumannii (e). All data are expressed as mean ± standard deviation as indicated by the error bars (n = 3). ***P < 0.001, **P < 0.05, Student’s t test.

Based on the results shown in Figure 5.3, the MBCs of the antibiotics when co-administered with S16, hereby denoted as MBCₐeff, were taken as the effective dose that resulted in at least a 2-log reduction in bacterial cell counts [39]. These values were computed and listed in Table 5.2. By comparing the MBCₐeff of each antibiotic with its original MBC when administered alone (see Appendix III, Supplementary Table 5.1 for the latter), the extent of reduction in the antibiotic concentration afforded as a result of the S16-antibiotic synergism was obtained (Table 5.2). Notably, while ampicillin on its own is ineffective against P. aeruginosa (MBC>5.86 mM), its activity can be improved by synergistic interactions with S16 which results in more than 16-fold reduction in MBC. Further, 3.7 to 6.5-fold reductions in MBC resulting from synergy with S16 were obtained for doxycycline and imipenem which possess poor efficacy against CMDR P. aeruginosa and CMDR A. baumannii, respectively, when administered alone. The ability of SNAPP S16 to synergize with imipenem, a type of carbapenem from the β-lactam class, is encouraging, as carbapenems have been viewed as the only available treatment option against many severe
infections caused by MDR bacteria and the emergence of carbapenem resistance among Gram-negative pathogens have been reported [29].

### Table 5.2. Fold Reduction of MBC Provided by Synergistic S16-Antibiotic Combinations

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>MBC in combination, MBC&lt;sub&gt;eff&lt;/sub&gt; (µM)</th>
<th>Fold reduction of MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amp</td>
<td>366.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CMDR <strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag&lt;sup&gt;+&lt;/sup&gt;, Dox</td>
<td>5.9&lt;sup&gt;a&lt;/sup&gt;, 148.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CMDR <strong>A. baumannii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ag&lt;sup&gt;+&lt;/sup&gt;, Imi</td>
<td>2.9&lt;sup&gt;a&lt;/sup&gt;, 88.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The MBC<sub>eff</sub> values for the antibiotics were taken as the antibiotic concentrations that resulted in at least a 2-log reduction in CFU/mL when co-administered with S16. <sup>b</sup> The MBC<sub>eff</sub> of S16 is equivalent to its MBC50.

#### 5.3.4 Mammalian Cell Toxicity Evaluation

The use of antibiotics has been linked to adverse side effects on the human body, such as neurotoxicity [40]. Silver ion (Ag<sup>+</sup>), while approved by the U.S. Food and Drug Administration (FDA) as a topical antimicrobial [7], has been widely acknowledged to display dose-dependent cytotoxicity which prohibits its systemic use. It was hypothesized that synergistic therapy could help to reduce the toxicity of such antibiotics as the effective antibiotic concentrations required would be reduced. As a proof-of-concept study, the S16-Ag<sup>+</sup> pair that was found to be synergistic against CMDR **A. baumannii** was selected as a model due to the inherent toxicity of Ag<sup>+</sup>. The viability of a model mammalian cell line – rat hepatoma H4IIE cells – was evaluated 90 min after incubation with S16, Ag<sup>+</sup> or a combination of S16 and Ag<sup>+</sup> via an apoptosis/necrosis assay (Figure 5.4). When treatment with either S16 or Ag<sup>+</sup> was applied at a dose equivalent to their MBC dose, H4IIE cell viability was reduced by 21 and 33%, respectively, compared to the untreated control. However, when the cells were treated with a cocktail of S16 and Ag<sup>+</sup> (at dosages equivalent to their MBC<sub>eff</sub>), negligible effect on cell viability was observed as compared to untreated cells. Since S16 and Ag<sup>+</sup> possess synergistic antimicrobial activities, the dosages of S16 and Ag<sup>+</sup> required for combination therapy are significantly reduced compared to the dosages required in monotherapy (i.e., 2-fold for S16 and 3.7-fold for Ag<sup>+</sup>). It was suggested that the
synergistic antimicrobial effects of SNAPPs with antibiotics could potentially be harnessed as a strategy to formulate highly potent but safe antimicrobial combinations for treatment.

**Figure 5.4. Effect of SNAPP S16-Ag\textsuperscript{+} synergistic pair on mammalian cell viability.**

Percent viability of rat hepatoma (H4IIE) cells after treatment with S16, Ag\textsuperscript{+} (AgNO\textsubscript{3}), and co-administration of S16 and Ag\textsuperscript{+} at the indicated concentrations. MBC = 1.6 µM for S16 and 10.6 µM for Ag\textsuperscript{+}; MBC\textsubscript{eff} = 0.8 µM for S16 and 2.9 µM for Ag\textsuperscript{+}. Note that the MBC and MBC\textsubscript{eff} values were taken based on the *in vitro* activities of the compounds against MDR A. baumannii. All data are expressed as mean ± standard deviation as indicated by the error bars (n = 4).

### 5.4 Chapter Summary

In conclusion, the ability of SNAPPs to synergize with antibiotics from different classes (*i.e.*, β-lactams, tetracyclines and Ag\textsuperscript{+}) against a range of clinically-relevant Gram-negative pathogens, including CMDR clinical isolates, was demonstrated in this chapter. Notably, excellent synergy was demonstrated by a model SNAPP with Ag\textsuperscript{+} against all bacterial species tested, which was attributed to the mechanistic similarities of both compounds in terms of ROS production and membrane disruption. Poor synergism, or in some cases antagonism, between SNAPPs and aminoglycosides was observed, possibly due to the competition between SNAPPs and the aminoglycoside for binding sites on the OM of
Gram-negative bacteria. The synergism between SNAPPs and ampicillin or gentamicin was found to be species-dependent, whereas highly synergistic interactions were observed for the combination of SNAPPs with either doxycycline or imipenem against the CMDR bacterial species. The efficacy of certain antibiotics against drug-resistant bacteria was restored through co-administration with SNAPPs, resulting in the reduction of antibiotic doses by 3.7 to 16-fold from their original effective dose. Furthermore, it was indicated that the use of SNAPPs as an antibiotic adjuvant could mitigate the toxic side-effects of certain antimicrobials. While further optimizations on the choice of SNAPP-antibiotic combinations as well as drug ratios and dosing regimens are required to boost the overall treatment efficacy, this study demonstrated the potential of the synergistic co-administration of SNAPPs and antibiotics as a novel treatment method against infections caused by drug-resistant Gram-negative bacteria.

5.5 References


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CHAPTER 6

Conclusions and Future Perspectives

6.1 Conclusions

This thesis sought to provide an innovative yet practical approach through macromolecular engineering to combat infections caused by multidrug-resistant (MDR) bacteria. The work was motivated by the fact that antimicrobial resistance has emerged as a serious global threat to human health, and yet the pipeline for new antibiotics is running dry. Inspired by a class of naturally-occurring molecules, known as antimicrobial peptides (AMPs), which form part of the innate immune system found in all living organisms, this thesis presented a novel platform technology, in the form of star-shaped peptide polymers, with the potential to serve as a better alternative to antibiotics in the fight against MDR bacteria.

Specifically, Chapter 2 documented the discovery and development of 16- and 32-arm star peptide polymers, named ‘Structurally Nanoengineered Antimicrobial Peptide Polymers’ (SNAPPs), as a new class of antimicrobial agents with efficacy against wild-type and MDR Gram-negative bacteria. The star polymers developed in this study possess random (co)peptide arms of lysine and valine. Using a mouse peritonitis model, SNAPPs were shown to possess \textit{in vivo} activity against \textit{A. baumannii}, including the colistin and MDR species. Notably, resistance development against SNAPPs was not observed in either wild-type or MDR \textit{A. baumannii}. SNAPPs also demonstrated high therapeutic indices, demonstrating their relative low toxicity towards mammalian cells. Through a comprehensive mechanistic study using a range of imaging and bioassay techniques, SNAPPs were found to possess a multi-modal mechanism of action, which differs substantially from that typically reported for AMPs. It was postulated that SNAPPs induced outer membrane (OM) destabilization, cytoplasmic membrane (CM) perturbations that lead to unregulated ion influx/efflux, and the apoptotic-like death pathway. On the contrary, most AMPs are known to translocate across the OM and act on the CM, causing cell death by CM disruption. The unique antimicrobial mechanism of SNAPPs could possibly account for (i) their superior and equipotent activity against all Gram-negative bacteria tested, including MDR species, as compared to AMPs, and (ii) the lack of observable resistance development to SNAPPs. Collectively, the findings from
this chapter established SNAPPs as promising drug candidates to combat Gram-negative pathogens, with the potential to address the discovery void in antimicrobial development.

**Chapter 3** presented a structure-activity relationship study to address several key research questions raised from the previous chapter. More specifically, the effects of peptide (co)polymer structure and star architecture were explored based on a polymer library consisting of 16- and 32-arm star polymers with random, block or homo peptide arms and their linear analogues. By using flow cytometry and conventional antimicrobial susceptibility assays, the star polymers with random (co)peptide arms of lysine and valine were found to be superior to those with block (co)peptide or pure poly(lysine) arms, in terms of their bacterial membrane disrupting and killing abilities. The star architecture was found to enhance the antimicrobial efficacy of random (co)peptides, which could be attributed to the ability of the star polymers to adopt an overall $\alpha$-helical conformation in a membrane-mimicking environment. On the other hand, polymer structure was demonstrated to have a less significant effect on polymer hemo- and mammalian cell compatibility.

Besides investigating the effects of polymer structure on antimicrobial activity, this thesis also sought to determine the nature of the interactions between the antimicrobial star peptide polymers and biomolecules present *in vivo*, and their effects on activity. In **Chapter 4**, a bio-nano interaction study was conducted by evaluating the efficacy of SNAPPs in different physiologically relevant environments. In simulated body fluid (SBF) which mimics the actual salt composition in blood plasma, divalent cations, in the form of magnesium and calcium ions, were found to inhibit the antimicrobial efficacy of SNAPPs by reducing their ability to disrupt bacterial OM and CM. This inhibitory effect was observed when SNAPPs were tested against *E. coli*, *K. pneumoniae* and *P. aeruginosa*. However, against *A. baumannii*, the membrane disruption ability of SNAPPs was only reduced initially and recovered within 25 min. Combined with the excellent *in vivo* efficacy of SNAPPs against MDR *A. baumannii* as reported in **Chapter 2**, this result highlighted the potential of SNAPPs as a promising lead in the treatment of *A. baumannii* infections. In blood serum, the presence of both salts and proteins had an antagonistic effect on the antimicrobial activity of SNAPPs. It was demonstrated that serum proteins could bind to SNAPPs, resulting in polymer aggregation and a subsequent decrease in the ability of SNAPPs to permeabilize bacterial membranes. As a proof-of-concept, a strategy to circumvent the inhibitory effect of divalent cations through chelating agent addition was also presented.
In Chapter 5, the potential of SNAPPs to be utilized as an antibiotic adjuvant was explored. Synergistic interactions between SNAPPs and a range of antibiotics were evaluated to determine if SNAPPs could potentiate antibiotic activity and possibly restore the efficacy of antibiotics that were ineffective when administered alone. Based on the Bliss and Highest Single Agent models, SNAPPs were shown to act in synergy with B-lactams (ampicillin and imipenem), a tetracycline (doxycycline) and silver ions. The synergistic effects were attributed to the ability of SNAPPs to disrupt bacterial membranes, thereby promoting the uptake of antibiotics. It was further demonstrated that SNAPPs can resensitize various antibiotic-resistant bacteria, including MDR clinical isolates, to antibiotics, such as ampicillin, doxycycline and imipenem. By co-administering a model SNAPP with the antibiotic, the effective antibiotic dose can be reduced by 3.7 to 16-fold. It was also shown that synergistic therapy, as demonstrated in this study, is able to attenuate mammalian cell toxicity associated with antimicrobial use.

Overall, the findings presented in this thesis substantiated the potential of SNAPPs as a new and effective class of Gram-negative antimicrobials and, hence, constitute a significant advancement in the field of antimicrobial development. The investigations outlined in this thesis are inter-disciplinary and provided fundamental understanding and insights on the bioactivity, chemical structural design, bio-nano interactions, and polymer-antibiotic interactions of SNAPPs – all of which will aid in the development of SNAPPs for clinical use.

6.2 Future Perspectives

6.2.1 Polymer Design and Optimization

Continuing from the star polymer platform established in this thesis, it is envisioned that the development of SNAPPs will be focused on increasing their antimicrobial potency and target specificity through advanced macromolecular engineering. A library of star-shaped peptide polymers with various configurations will be synthesized to enable systematic optimization of the polymer structure for improved antibacterial activity. Variations will be made to (i) peptide valency or the number of star arms, (ii) the length of each arm, (iii) the lysine-to-valine ratio, and (iv) the type of amino acids or functional groups used. The architecture of SNAPPs could be extended beyond the star morphology to other complex macromolecular architectures, such as brush and hyperbranched nanostructures. It is anticipated that these changes would affect the overall polymer size, peptide secondary structure and charge density, which may impact the antimicrobial activity and
biocompatibility of SNAPPs. For example, increasing arm length is likely to affect antimicrobial activity. Previous studies on linear antimicrobial polymers have shown that activity can be altered (increased or decreased, depending on polymer type and composition) by increasing the polymeric chain length [1,2]. Each modification to the polymeric system will be tested \textit{in vitro} to provide feedback and aid in the optimization of next-generation SNAPPs.

In this thesis, the star peptide polymers were designed to possess a net positive charge to promote interaction with bacterial membranes, which are negatively charged, and not mammalian cell membranes, which are zwitterionic. While this approach enabled the broad spectrum efficacy of SNAPPs, it is evident from Chapters 2 and 3 that non-specific binding of the polymers with mammalian cells still exist and remains as a major challenge. Work is needed to engineer SNAPPs such that greater specificity for bacteria can be achieved. A potential strategy is through the use of targeting ligands, such as peptides [3], lectins [4], antibodies [5], or aptamers [6], conjugated to the periphery of SNAPPs. Besides improving SNAPP biocompatibility, this might enhance the binding of SNAPPs to bacteria even in environments that possess high protein concentrations. The impact of the use of highly specific targeting moieties on antimicrobial resistance development would also need to be investigated.

6.2.2 Further Exploration of the Precise Antimicrobial Mechanism

While the results contained in this thesis offered insights on the mode of action of SNAPPs, work is still needed to obtain a more in-depth understanding. Specifically, the sequence of events that takes place within a bacterial cell following SNAPP administration, the precise point of cell death and the impact of the star architecture on the antimicrobial mechanism need to be investigated in greater detail. Other questions to be resolved include: the interaction of SNAPPs with the peptidoglycan layer, effect(s) of SNAPP treatment on the antibiotic resistance machinery of MDR bacteria, and the immunomodulatory behaviours of SNAPPs. In all potential assays to be conducted, controls in the form of representative AMPs and/or antibiotics need to be incorporated to facilitate a side-by-side comparison of SNAPPs with other antimicrobials.

6.2.3 Translating from Bench to Bedside

In Chapter 2, the \textit{in vivo} efficacy of SNAPPs was demonstrated through a mouse peritonitis model against wild-type and MDR \textit{A. baumannii} infections. As an extension to
this thesis, the lead SNAPP compounds will need to be tested for their in vivo effectiveness against other Gram-negative bacterial infections, such as *K. pneumoniae* and *P. aeruginosa*, as well as in other local and systemic infection models, such as the mouse urinary tract, pneumonia and skin infection models. Future work will also be focused on establishing the toxicity profile of SNAPPs using healthy, infection-free mice, by analysing the effects of SNAPP treatment on blood chemistry and organ functions. Additionally, through the use of small animal models, the pharmacokinetics, biodistribution and stability of SNAPPs would need to be profiled and compared across different routes of administration. Taken together, these information would prove valuable information in advancing SNAPPs to clinical trials.

### 6.3 References

**CHAPTER 7**

**Experimental**

This chapter describes the materials, characterisation methods and detailed experimental procedures utilised in all the work presented in this thesis.

### 7.1 Materials

H-Lys(Z)-OH (>99%, Fluka), DL-Valine (>99%, Acros Organics), sodium chloride (NaCl, Chem-Supply), potassium chloride (KCl, Chem-Supply), sodium phosphate dibasic (Na$_2$HPO$_4$, Chem-Supply), potassium phosphate monobasic (KH$_2$PO$_4$, 99%, Aldrich), sodium bicarbonate (NaHCO$_3$, ≥99.5%, Aldrich), potassium phosphate dibasic trihydrate (K$_2$HPO$_4$·3H$_2$O, ≥99.0%, Aldrich), magnesium chloride hexahydrate (MgCl$_2$·6H$_2$O, 99.0-102.0%, Aldrich), hydrochloric acid (HCl, Chem-Supply), calcium chloride (CaCl$_2$, ≥99.9%, Aldrich), sodium sulphate (Na$_2$SO$_4$, ≥99.0%, Aldrich), Tris base (NH$_2$C(CH$_2$OH)$_3$, Aldrich), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, 99.0-101.0%, Aldrich), N-phenyl-1-naphthylamine (NPN, 98%, Aldrich), bovine serum albumin (BSA, Bovogen Biologicals), sucrose (Univar), D-(+)-glucose solution (100 g/L, Aldrich), diethyl ether (Chem-Supply), acetonitrile (Univar), generation 2.0 poly(amido amine) dendrimer (G2 PAMAM, 8.95% w/w in water) (Dendritech), generation 3.0 poly(amido amine) dendrimer (G3 PAMAM, 8.84% w/w in water) (Dendritech), 4-methylbenzylamine (97%, Aldrich), bis(trichloromethyl)carbonate (triphosgene, 99%, Aldrich), paraformaldehyde (Aldrich), trifluoroacetic acid (TFA) (99%, Aldrich), hydrobromic acid (33% in acetic acid) (Aldrich), pentane (anhyd., >99%, Aldrich), dimethyl sulfoxide (DMSO, Aldrich), N,N-dimethylformamide (DMF, anhyd., Acros Organics), Spectra/Por® molecular porous membrane tubing 8000 MWCO (Spectrum Laboratories, Inc.), poly-D-lysine hydrobromide (70-150 kDa, Aldrich), Hanks’ Balanced Salt solution (HBSS, with sodium bicarbonate, without phenol red, Aldrich), penicillin-streptomycin (Aldrich), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE, Avanti Polar Lipids), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG, Avanti Polar Lipids), lucigenin (N,N'-dimethyl-9,9’-biacridinium dinitrate, Aldrich), fluorescein isothiocyanate-dextran (4 kDa, Aldrich), rhodamine isothiocyanate-dextran (70 kDa, Aldrich), thiourea (>99%, Aldrich), ampicillin (Aldrich), gentamicin solution (50 g/L, Aldrich), imipenem monohydrate (Aldrich), silver nitrate (AgNO$_3$, >99%, Aldrich), and doxycycline hyclate (>98%, Aldrich) were used as
received. THF (99%, Lab Scan) was distilled from sodium benzophenone ketal under argon. Dimethylsulfoxide-$d_6$ (DMSO-$d_6$) (99.9%) was purchased from Cambridge Laboratory Isotopes and used as received. RPMI-1640 medium without L-glutamine (GIBCO Cat. No. 21870), Dulbecco’s Modified Eagle Medium (DMEM, GIBCO Cat. No. 11995), fetal bovine serum (FBS, GIBCO Cat. No. 10099), GlutaMAX™ supplement (100x, GIBCO Cat. No. 35050), antibiotic-antimycotic (100x, GIBCO Cat. No. 15240), MEM non-essential amino acids (100x, GIBCO Cat. No. 11140), Dulbecco’s Phosphate Buffered Saline (DPBS, GIBCO 14190), 0.05% trypsin-EDTA (1X, GIBCO Cat. No. 25300), Alexa Fluor® 488 carboxylic acid succinimidyl ester, FM® 4-64FX, SYTO® 9 green fluorescent nucleic acid stain, and PI were purchased from Invitrogen and used as received. Defibrinated horse and sheep blood were obtained from Commonwealth Serum Laboratories (CSL) Melbourne. Todd-Hewitt Broth (CM0189), Mueller-Hinton Broth (MHB) (CM0405), Blood Agar Base No. 2 (CM0271), and Yeast Extract (LP0021) were purchased from Oxoid. Bacto™ Tryptone, and Bacto™ Agar were purchased from BD Biosciences. Ultra-pure lipopolysaccharide (LPS) from *Escherichia coli O111:B4* was purchased from Invivogen. Vybrant® Apoptosis Assay Kit #4 (YO-PRO®-1/PI, Invitrogen) was used to perform the apoptosis/necrosis assay. BacLight Bacterial Membrane Potential Kit (Invitrogen) was used to conduct the membrane potential assay. RNeasy Protect Bacteria Mini Kit (Qiagen), TURBO DNA-free kit (Ambion), iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories), and iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) were used for RNA extraction and reverse transcriptase PCR analysis for programmed cell death pathways. CellROX® Orange Reagent (Invitrogen) was used to perform the reactive oxygen species (ROS) production assay. 96-well cell culture plates and T175 cell culture flasks (Corning) were used for cell culture. 8-Well Nunc™ Lab-Tek™ Chambered Coverglass (Thermo Scientific) was used to contain samples for imaging with 3D-SIM.

### 7.2 Characterization Methods

#### 7.2.1 Circular Dichroism (CD)

For Chapter 3, CD spectra were recorded at 25 °C on an Aviv Biomedical CD Spectrometer Model 410 with stopped flow unit add-on. A glass cuvette with a path length of 1 mm was used. Spectra were generated from 195 to 250 nm wavelengths at 0.5 nm intervals, 4 s averaging time, 0.333 s settling time, and 1 nm bandwidth. Polymers were dissolved to a final
concentration of 0.2 µg/mL in RO water with 0%, 20%, 50%, and 80% TFE. The spectra were plotted as mean residue ellipticity, [θ], against wavelength.

7.2.2 Cryo-Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM was used to image bacterial cell morphology in Chapter 2. The mounting and plunge freezing of samples were performed as previously described [1]. Imaging was conducted using a FEI Tecnai G2 TF30 (FEI company) that was operated at 200 kV and equipped with a Gatan US1000 2k × 2k CCD camera (Gatan). Micrographs were recorded under low-dose conditions with underfocus values of 4-6 µm.

7.2.3 Dynamic Light Scattering (DLS)

DLS measurements (Chapters 2 and 3) were performed on a Malvern Zetasizer Nano ZS with a 4.0 mW He-Ne laser (633 nm) at an angle of 173° and a temperature of 25 ± 0.1°C. Initial sample concentrations of 1 mg/mL in either water purified by reverse osmosis, MEM or MHB were used and serial dilutions were performed until stable spectra were obtained. All sample solutions were filtered using 0.45 μm syringe filters.

7.2.4 Flow Cytometry

Bacterial cell sample analysis was performed using a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter) equipped with a 100 W stabilized mercury arc lamp (optimised at 366, 405 and 435 nm) and a 488 nm diode laser. For bacterial cell counting and viability measurements (Chapters 2-5), the fluorescence from SYTO® 9 was measured through a 525-nm band-pass filter (Fluorescent Channel 1, FL-1), and the red emission of propidium iodide (PI) was measured with a 670-nm long pass filter (Fluorescent Channel 3, FL-3). For the membrane potential assay in Chapter 2, fluorescence signals were collected in FL-1 and FL-3 to determine the ratio between polarised/hyperpolarised and depolarised cells. Fluorescence from the CellROX® Orange Reagent was measured on FL-3 to determine the extent of reactive oxygen species (ROS) production (Chapter 2). All data were analyzed using the Cell Lab Quanta SC software.

Mammalian cell sample analysis for the apoptosis/necrosis assay (Chapters 2, 3 and 5) was performed on a Cytomics FC500 MPL flow cytometer (Beckman Coulter) equipped with a 20 mW argon ion laser operating at 488 nm and a 25 mW red diode laser with a nominal wavelength of 635 nm. The fluorescence signals from YO-PRO®-1 and PI were collected in FL-1 and FL-3. The MXP software was used for data acquisition and analysis, respectively.
7.2.5 Fluorescence Microscopy

Super-resolution fluorescence imaging for Chapter 2 was performed using three dimensional-structured illumination microscopy (3D-SIM) implemented on the DeltaVision Optical Microscope eXperimental (OMX) V4 Blaze imaging system (Applied Precision, a GE Healthcare Company). Briefly, 488 and 568 nm lasers (for Alexa Fluor® 488 and FM® 4-64FX, respectively) were used to provide wide-field illumination and multi-channel images, which were captured on two PCO Edge scientific CMOS cameras (each dedicated to a specific channel) with acquisition rates of up to 400 fps. Data capture used an Olympus PlanApo N 60× 1.42 NA oil objective and excitation and emission filter sets of 528/48 for Alexa Fluor® 488 and 609/37 for FM® 4-64FX. The immersion oil 1.514 (GE Healthcare Company) was used. The DeltaVision OMX Master Control Software was used for instrument control. All raw images were reconstructed using SoftWorX 4.0 (Applied Precision). Image analysis was performed using Fiji, a distribution of ImageJ.

7.2.6 Gel Permeation Chromatography (GPC)

Aqueous-GPC analysis (Chapters 2 and 3) was performed on a Shimadzu liquid chromatography system equipped with a Shimadzu RID-10 refractometer (λ = 633 nm), using three Waters Ultrahydrogel columns in series ((i) 250 Å porosity, 6 µm diameter bead size; (ii) and (iii) linear, 10 µm diameter bead size), operating at 60 °C. The eluent was Milli-Q water containing 20% v/v acetonitrile and 0.1% w/v TFA (0.5 mL/min). The molecular weight characteristics of the analytes were determined with reference to a conventional column calibration with narrow molecular weight distribution poly(ethylene glycol) standards (Polymer Standards Service GmbH). All samples for GPC analysis were prepared at a concentration of 10 mg/mL and were filtered through 0.45 µm nylon filters prior to injection.

7.2.7 Nuclear Magnetic Resonance (NMR) Spectroscopy

$^1$H NMR spectroscopy (Chapters 2 and 3) was performed at room temperature using a Varian Unity400 (400 MHz) spectrometer with the deuterated solvent as reference and a sample concentration of ca. 10 mg/mL.

7.3 Statistical Analysis

Statistical analysis was performed using a one-way classification of ANOVA and student’s $t$-test (two-tailed), where differences were regarded as statistically significant with probability $P>0.05$. 

164
7.4 General Experimental Methods

Synthesis of L-lysine(Z)-NCA (Lys NCA). H-Lys(Z)-OH (1.24 g, 4.42 mmol) was added to anhydrous THF (15 mL) in an oven-dried two-neck round bottom flask under argon. Triphosgene (525 mg, 1.77 mmol) was dissolved in anhydrous THF (2 mL) and added to the H-Lys(Z)-OH suspension. The mixture was heated at 50 °C for 30 min with continuous stirring. After the clear solution was sparged with argon to remove any dissolved HCl and allowed to cool to room temperature, two-thirds of the solvent was removed in vacuo and the resulting residue was recrystallized from 1:1 ethyl acetate:hexane (20 mL) 2 times. After the final recrystallization step, the resulting white solid was washed with anhydrous pentane (30 mL × 2) and dried at ambient temperature in vacuo to afford Lys NCA, 1.097 g (81%). The inclusion of a HCl scavenger, in the form of α-pinene, in the synthetic process was found to yield a similar product and hence was regarded as redundant. $^1$H NMR (400 MHz, $d_6$-DMSO) δ$_H$ 1.23-1.37 (m, $\gamma$-CH$_2$, 2H), 1.37-1.45 (m, $\delta$-CH$_2$, 2H), 1.60-1.80 (m, $\beta$-CH$_2$, 2H), 2.94-3.02 (m, e-CH$_2$, 2H), 4.40-4.43 (m, $\alpha$-CH, 1H), 5.00 (dd, C$_6$H$_5$CH$_2$, 2H), 6.90 (s, cyclic NH, 1H), 7.30-7.39 (m, C$_6$H$_5$, 5H).

Synthesis of DL-valine-NCA (Val NCA). DL-valine (1.24 g, 10.58 mmol) was dissolved in anhydrous THF (20 mL) in an oven-dried two-neck round bottom flask under argon. Triphosgene (1.26 g, 4.23 mmol) was dissolved in anhydrous THF (5 mL) and added to the DL-Val-THF suspension. The mixture was heated at 50 °C for 30 min with continuous stirring. After the clear solution was sparged with argon to remove any dissolved HCl and allowed to cool to room temperature, two-thirds of the solvent was removed in vacuo and the resulting residue was recrystallized from 1:1 ethyl acetate:hexane (20 mL) 2 times. After the final recrystallization step, the resulting white solid was washed with anhydrous pentane (30 mL × 2) and dried at ambient temperature in vacuo to afford Val NCA, 1.29 g (85%). The inclusion of a HCl scavenger, in the form of α-pinene, in the synthetic process was found to yield a similar product and hence was regarded as redundant. $^1$H NMR (400 MHz, $d_6$-DMSO) δ$_H$ 0.91 (dd, CH$_3$, 6H), 2.00-2.12 (m, CH, 1H), 4.32 (dd, cyclic CH, 1H), 9.06 (s, cyclic NH, 1H).

General procedure for deprotection of peptide polymers. The peptide polymer was dissolved in TFA (200 mg/mL) and 33% HBr in acetic acid was then added (20 mL/g peptide polymer). After 24 h stirring at room temperature, the mixture was precipitated into diethyl ether (10 times the volume of the reaction). The precipitate was isolated via centrifugation,
redissolved in hydrochloric acid solution (0.2 M, 0.2 mL/mg peptide polymer), and dialyzed against RO water for 4 days. The dialyzed solution was lyophilized to obtain the deprotected peptide polymer.

**Bacterial cell culture.** Freeze-dried cultures of *Escherichia coli* (*E. coli*, ATCC 25922), *Klebsiella pneumoniae* (*K. pneumoniae*, ATCC 13883), *Acinetobacter baumannii* (*A. baumannii*, ATCC 19606), colistin- and multi-drug resistant (CMDR) *A. baumannii* (FADDI-AB156), CMDR *P. aeruginosa* (FADDI-PA067) and *Staphylococcus aureus* (*S. aureus*, ATCC 29213) were grown aerobically and maintained by passage at ambient temperature on horse blood agar (10% v/v defibrinated horse blood, 4.4% w/v Oxoid Blood Agar Base No. 2). *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 47085) were cultured in a similar fashion, except at 37 °C. Freeze-dried cultures of *Streptococcus mutans* (*S. mutans*, Ingbritt strain) were grown anaerobically and maintained by passage at 37 °C on Todd Hewitt agar (3.6% w/v Oxoid Todd-Hewitt Broth, 1.5% w/v sucrose, 1.5% w/v Bacto™ Agar, 0.8% w/v Oxoid Yeast Extract). For *E. coli*, *K. pneumoniae*, *P. aeruginosa*, CMDR *P. aeruginosa*, *A. baumannii*, CMDR *A. baumannii* and *S. aureus*, overnight cultures were made from transferring a colony (ca. half a loop) from the agar plates to culture tubes containing sterilized Luria-Bertani broth (LB, 1% w/v Bacto™ Tryptone, 1% w/v NaCl, 0.5% w/v Oxoid Yeast Extract) (20 mL). Bacterial cultures were incubated overnight at 37 °C with aeration and without agitation, with the exception of CMDR *P. aeruginosa* which was cultured at 37 °C with aeration and agitation (150 rpm). On the next day, for *E. coli*, *K. pneumoniae*, *P. aeruginosa*, CMDR *P. aeruginosa* and *S. aureus*, small aliquots (i.e., 0.5-2 mL) were taken from the culture tubes, further diluted with LB (20 mL), and incubated for 3-4 h at 37 °C with aeration before use. All bacterial cultures were cultured without agitation, with the exception of *P. aeruginosa* and CMDR *P. aeruginosa* which were cultured with shaking at 150 rpm. For *A. baumannii*, an aliquot of 0.5 mL was taken from the overnight culture tube, further diluted with LB (200 mL), and incubated overnight at 37 °C with aeration before use. With regards to *S. mutans*, several colonies (ca. half a loop) from the agar plates were transferred to culture tubes containing sterilized Todd Hewitt broth (3.6% w/v Oxoid Todd-Hewitt Broth, 1.5% w/v sucrose, 0.8% w/v Oxoid Yeast Extract) (20 mL). The cultures were incubated overnight at 37 °C in the anaerobic chamber. After 24 h, a small aliquot (i.e., 0.5 mL) was taken from the culture tubes, further diluted with media (ca. 200 mL), and incubated overnight at 37 °C in the anaerobic chamber before use.
**Bacterial cell counting.** A Cell Lab Quanta SC MPL flow cytometer was used to count the number of bacterial cells prior to use in assays. Cells were diluted with saline using an appropriate dilution factor and incubated with Syto® 9 and PI (i.e., 1 mL cell solution to 1 µL of each dye). Syto® 9 stains the nucleic acids in all cells, while PI stains the nucleic acids in cells with damaged membranes. Using the Cell Lab Quanta SC software, the number of viable cells/mL (Syto® 9-positive, PI-negative) was obtained.

**Measurement of minimum disruptive concentrations (MDC).** A dilution series of each peptide polymer was made by diluting peptide polymer stock in sterilized Mueller-Hinton broth (MHB, see Supplemental Experimental Procedures for composition) for *Escherichia coli*, Luria broth (LB, see Supplemental Experimental Procedures for composition) for *Staphylococcus aureus* or Todd Hewitt broth (see Supplemental Experimental Procedures for composition) for *Streptococcus mutans* to a desired concentration range with a final volume of 100 µL in each well of a 96-well plate. Bacterial cells (which gave an optical density reading of ca. 0.7 at 650 nm for *E. coli* and *S. aureus* and ca. 1.8 at 650 nm for *S. mutans*) were diluted to $2.5 \times 10^6$ cells/mL and 100 µL of the bacteria solution was added to each well. The 96-well plate was then incubated at 37 °C for 90 min. A 50 µL aliquot was taken from each well, transferred to a second 96-well plate and 100 µL of saline and dye mixture (i.e., saline with 0.1% of SYTO® 9 and 0.1% of PI) was added. Each well in the second 96-well plate was analyzed with a Cell Lab Quanta SC MPL flow cytometer to determine the % of cells with intact membranes and cells with compromised membranes for each polymer at each concentration. Positive controls containing cells alone were incorporated. Percentage of cells with intact membranes was plotted against polymer concentration and linear regression analysis was used to determine the lowest concentration (MDC) at which all of the cells had their membranes disrupted. A minimum of two independent experiments (biological replicates) of the assay were conducted and two technical replicates were used in each experiment for each bacterium, compound, and concentration. Data is expressed as mean ± standard deviation (SD) of the biological replicates and analysed using student’s *t*-test.

**Measurement of minimum bactericidal concentrations (MBC).** A dilution series of each compound was made by diluting test compound stock in media to a desired range of concentrations and a final volume of 100 µL in each well of a 96-well plate. Bacterial cells (which gave an optical density reading of ~0.7 at 650 nm for *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*, ~0.5 at 650 nm for *A. baumannii* and ~1.8 at 650 nm for *S.
mutans) were diluted to $2.5 \times 10^6$ cells/mL in media and 100 µL of the bacteria solution was added to each well. The 96-well plate was then incubated at 37 °C for 90 min. For each well, microbial solution was diluted with saline (0.9% NaCl solution) using an appropriate dilution factor and placed on an agar plate (identical to that used for bacteria culture). For E. coli, K. pneumoniae, A. baumannii, and S. aureus, the agar plates were incubated overnight at room temperature, and then at 37 °C with aeration for 2 h. For P. aeruginosa and S. mutans, the agar plates were incubated at 37 °C, with the former being incubated overnight with aeration and the latter in an anaerobic chamber for 48 h. The number of colony-forming units (CFU) was counted and expressed as CFU/mL. Positive controls consisting of cells without any treatment were used. Concentration-killing curves were plotted with CFU/mL as a function of compound concentration and linear regression analysis was used to determine the lowest concentration (MBC) at which the CFU/mL becomes zero (Chapter 2, Supplementary Figure 2.9). A minimum of two independent experiments (biological replicates) of the assay were conducted and two technical replicates were used in each experiment for each bacterium, compound, and concentration. Data is expressed as mean ± standard deviation (SD) of the biological replicates and analyzed using student’s t-test. Note that in Chapter 2, for E. coli, K. pneumoniae, P. aeruginosa, and A. baumannii, two sets of experiments were performed, either using a nutritionally-rich medium (MHB, 3.8% w/v Oxoid Mueller-Hinton Agar) or minimal essential medium (MEM, 136.9 mM NaCl, 10.1 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, 0.2% w/v D-(+)-glucose). For S. aureus and S. mutans, the assays were conducted in nutritionally-rich media only, which were Luria-Bertani broth and Todd-Hewitt broth, respectively.

Measurement of minimum inhibitory concentrations (MIC). The MICs of the PAMAM dendrimers were determined using a broth microdilution method. After the preparation of a dilution series of each compound and the addition of bacterial cells, the optical density readings of each well at 630 nm were measured as a function of time using a microplate reader (Multiskan Ascent, Pathtech Pty. Ltd.). Positive controls containing cells alone were incorporated. Optical density was plotted against polymer concentration and linear regression analysis was used to determine the lowest concentration (MIC) at which the optical density reading becomes zero. A minimum of two independent experiments (biological replicates) of the assay were conducted and two technical replicates were used in each experiment for each bacterium, polymer, and concentration. Data is expressed as mean ± standard deviation (SD) of the biological replicates and analyzed using student’s t-test.
In Chapter 4, nine types of media were used for the assay: minimal essential medium (MEM; 1× phosphate-buffered saline with 1× MEM non-essential amino acids and 2 g/L D-(+)-glucose), simulated body fluid (SBF; prepared according to the protocol reported by Kokubo et al. [2] and supplemented with 1× MEM non-essential amino acids and 2 g/L D-(+)-glucose), modified SBF (mSBF; prepared in a similar manner to SBF but without the addition of CaCl₂ and MgCl₂·6H₂O), SBF without CaCl₂ (prepared in a similar manner to SBF but without the addition of CaCl₂), SBF without MgCl₂·6H₂O (prepared in a similar manner to SBF but without the addition of MgCl₂·6H₂O), 10% serum (10% v/v FBS in MEM), 50% serum (50% v/v FBS in MEM), 0.22 mg/mL BSA in MEM, and 1.1 mg/mL BSA in MEM.

**Hemolysis assay.** Fresh sheep red blood cells (RBCs) were diluted 1 in 20 in PBS (pH 7.4), pelleted by centrifugation, and washed three times in PBS (1000 g, 10 min). The RBCs were counted using a cell counter (Coulter Particle Counter Z series, Beckman Coulter) and diluted to a final concentration of 2 × 10⁷ cells/mL. 100 µL aliquots of the RBC solution were seeded into a V-bottomed 96-well plate containing 100 µL of test compound solution of varying concentrations (4-2000 µg/mL) and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C for 2 h. Following incubation, the 96-well plate was centrifuged (1000 g, 10 min) and aliquots (100 µL) of supernatant were transferred to a flat-bottomed 96-well plate. Hemoglobin release upon lysis of the RBCs was monitored at 405 nm using a microplate reader (PerkinElmer 1420 Multilabel Counter VICTOR³). Positive and negative controls for hemolysis were taken as RBC lysed with 0.5% Triton X-100 (1:1 v/v) and RBC suspension in PBS, respectively. The percentage of hemolysis was calculated using the following formula:

\[
\text{% Hemolysis} = \left( \frac{A_{405 \text{ test sample}} - A_{405 \text{ negative control}}}{A_{405 \text{ positive control}} - A_{405 \text{ negative control}}} \right) \times 100
\]

The percentage hemolysis was plotted against peptide polymer concentration and linear regression analysis was used to determine the hemolytic concentration needed to lyse 50% (HC₅₀) of RBCs. Two independent runs of the assay were conducted and two replicates were used in each run for each compound and concentration.

**Mammalian cell culture.** Human embryonic kidney cells (HEK293T) were cultivated in ‘complete’ RPMI-1640 medium (supplemented with 5% FBS, 1× GlutaMAX™, 1× antibiotic-antimycotic, and 1× MEM non-essential amino acids) in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells were seeded in a T75 flask (ca. 3 × 10⁶ cells).
cells/ml) and passaged twice a week prior to performing the subsequent cell viability studies. Rat hepatoma cells (H4IIE) were cultivated in DMEM medium (supplemented with 10% FBS, 1× GlutaMAX™, and 1× penicillin-streptomycin) in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells were seeded in a T75 flask (ca. 3 × 10⁶ cells/ml) and passaged twice a week prior to performing the subsequent cell viability studies.

**Apoptosis/necrosis assay.** Adherent HEK293T or H4IIE cells (obtained from the ATCC, and throughout the course of the study were checked for mycoplasma contamination using Mycoplasma stain kit, Myc1, Aldrich) were grown to 80% confluence and trypsinized prior to assay. HEK293T and H4IIE cells were chosen for this study as they are standard cell lines used in toxicity studies. Cells were diluted 1:2 with ‘complete’ medium (RPMI-1640 for HEK293T cells or DMEM for H4IIE cells) and seeded in a 24-well plate (1 mL per well). The cells were incubated at 37 °C in 5% CO₂ for 24 h until ca. 95% confluence. The medium was removed. Varying concentrations of test compound (4 to 128 µg/mL) were prepared and 200 µL aliquots of each were added to the cells, after which the cells were incubated at 37 °C in 5% CO₂ for 90 min. The cells were then harvested and all well contents were transferred to round-bottomed polypropylene tubes (5 mL). The cells were washed with cold DPBS, then stained with YO-PRO®-1 and PI (0.2 mL from a stock solution, whereby both dyes were diluted 1:1000 in cold DPBS, per well), and incubated on ice for 20 to 30 min. The cells were analyzed by flow cytometry (Cytomics FC 500 MPL System). Standard compensation was performed using single-colour stained cells. Negative controls using untreated cells were included. Two independent runs of the assay were conducted and two replicates were used in each run for each test compound and concentration.

### 7.5 Specific Experimental Procedures for Chapter 2

**Synthesis of poly(Z-L-lysine-r-DL-valine)armPAMAM-(NH₂)₁₆,core star peptide polymer S₁₆₂.** Lys NCA (1.3 g, 4.19 mmol) and Val NCA (0.3 g, 2.1 mmol) were dissolved in anhydrous DMF (16 mL) and added via syringe to PAMAM-(NH₂)₁₆ (dried, 43 mg, 13.1 µmol) dissolved in anhydrous DMF (1 mL). After stirring for 24 h under argon, n-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated peptide polymer solution into diethyl ether (3 × 40 mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded (PZLL-r-PVal)armPAMAM-(NH₂)₁₆,core star peptide polymer S₁₆₂ as an off-white solid, 1.21 g (90%). ¹H NMR (400 MHz, d₆-DMSO) δH 0.67-0.89 (b, CH₃, 6H), 1.11-1.77 (b, γ-CH₂ + δ-CH₂ + β-CH₂, 6H), 1.84-2.00 (b,
CH, 1H), 2.78-3.00 (b, ω-CH₂, 2H), 4.06-4.40 (b, α-CH, 1H), 4.90-5.00 (b, C₆H₅CH₂-, 2H), 7.00-7.44 (b, C₆H₅-, 5H), 7.60-8.30 (b, NH, 1H).

Synthesis of poly(Z-L-lysine-r-DL-valine)₃₂,core star peptide polymer S₃₂Z. Lys NCA (1.3 g, 4.19 mmol) and Val NCA (0.3 g, 2.1 mmol) were dissolved in anhydrous DMF (16 mL) and added via syringe to PAMAM-(NH₂)₃₂ (dried, 43 mg, 13.1 µmol) dissolved in anhydrous DMF (1 mL). After stirring for 24 h under argon, n-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated peptide polymer solution into diethyl ether (3 × 40 mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded (PZLL-r-PVal)₃₂,core star peptide polymer S₃₂Z as an off-white solid, 1.15 g (85%).

1H NMR (400 MHz, d₆-DMSO) δH 0.67-0.89 (b, CH₃, 6H), 1.11-1.77 (b, γ-CH₂ + δ-CH₂ + β-CH₂, 6H), 1.84-2.00 (b, CH, 1H), 2.78-3.00 (b, ω-CH₂, 2H), 4.06-4.40 (b, α-CH, 1H), 4.90-5.00 (b, C₆H₅CH₂-, 2H), 7.00-7.44 (b, C₆H₅-, 5H), 7.60-8.30 (b, NH, 1H).

Synthesis of linear poly(Z-L-lysine-r-DL-valine) peptide polymer L₇Z. Lys NCA (0.5 g, 1.63 mmol) and Val NCA (117 mg, 0.82 mmol) were dissolved in anhydrous DMF (6 mL) and added via syringe to 4-methylbenzylamine (10.3 µL, 80.9 µmol). After stirring for 24 h under argon, n-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated peptide polymer solution into diethyl ether (3 × 40 mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded linear PZLL-r-PVal peptide polymer L₇Z as an off-white solid, 420 mg (81%).

1H NMR (400 MHz, d₆-DMSO) δH 0.67-0.89 (b, CH₃, 6H), 1.11-1.77 (b, γ-CH₂ + δ-CH₂ + β-CH₂, 6H), 1.84-2.00 (b, CH, 1H), 2.78-3.00 (b, ω-CH₂, 2H), 4.06-4.40 (b, α-CH, 1H), 4.90-5.00 (b, C₆H₅CH₂-, 2H), 7.00-7.44 (b, C₆H₅-, 4H), 7.60-8.30 (b, NH, 1H).

Synthesis of AMPs (ovispirin, magainin II and melittin). Ovispirin (NH₂-KNLRRRIIRKIIHIKKY-G-COOH), magainin II (NH₂-GIGKFLHSAKKFGKAFVGEIMNS-CONH₂) and melittin (NH₂-GIGAVLKVLTTGLPALISWIKKRQQQ-CONH₂) were chemically synthesized on a CEM Liberty microwave peptide synthesizer (Ai Scientific, Victoria, Australia). The peptide-resins were assembled from Fmoc-Rink-AM SURE™ Resin in the Fmoc/TBu mode of synthesis. For a 0.1 mmol reaction scale, Fmoc-deprotection was performed in two stages by initial treatment with 20% piperidine/0.1 M HOBT/DMF (v/v, 7 ml) under microwave radiation for 30 s (40 W, 40 °C), followed by filtration and a second addition of the above solution (45 W, 75 °C; 3 min). The peptide-resins were then rinsed with
DMF (4 × 7 ml). Acylation, where required, was achieved by the addition of a solution containing amino acid (5 eq, relative to reaction scale), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (5 eq) and N,N-diisopropylethylamine (DIEA) (10 eq) in DMF/NMP (7:1, v/v; 4 ml) to the Nα-deprotected peptide-resin and the mixture agitated under microwave radiation for 10 min (30 W, 75 °C, vessel under external chilled air flow). Dichloromethane (DCM) (5 × 2 min) was used to rinse the peptide-resins prior to the cleavage step. The peptide was cleaved from the resin support by the addition of TFA/triisopropylsilane (TIPS)/thioanisole/phenol/water (90:2.5:2.5:2.5:2.5, % v/v/v/v/v; 5 ml) for 2.5 h, after which the combined cleavage filtrates were evaporated under nitrogen flow and the crude product was isolated by precipitation in cold ether (4 × 30 ml).

The crude peptide was purified using an Agilent 1200 series liquid chromatograph instrument (Agilent, NSW, Australia) equipped with a UV detector (model G1316A) and a Zorbax 300 SB-C18 reversed phase column (9.4 mm × 25 cm). Crude peptide analysis was achieved using a linear acetonitrile gradient in 0.1% TFA at a flow rate of 4 mL/min (linear gradient of 0 to 54% CH₃CN over 15 min). Analysis of the purified peptide was performed using an Esquire HCT electrospray ionization-mass spectrometry system (Bruker Daltronics, NSW, Australia).

**Resistance studies.** The method used was adapted from Gullberg *et al* [3]. Overnight cultures of *A. baumannii* cells (ATCC 19606 or FADDI-AB156) in LB broth were obtained from independent colonies grown on horse blood agar. The cells were then serially passaged by 400-fold dilution in 1 mL batch cultures every 24 h for 600 generations (ca. 25 generations of growth per serial passage) in MHB containing 1/10 of the MBC of S16 (for both strains). After every 100 generations of growth, the MBCs of S16 were obtained using cells that were serially passaged in the presence of the antimicrobial agent. As a control, MBCs were also obtained using cells serially passaged in fresh MHB alone.

**In vivo efficacy of SNAPP S16.** All experiments involving animals were performed according to protocols approved by the University of Melbourne Biochemistry and Molecular Biology, Dental Science, Medicine, Microbiology and Immunology, and Surgery Animal Ethics Committee (Project number 1513489). 10 to 14-week-old female C57BL/6 mice (weighing 23.2 ± 1.7 g, animals under 20 g were not used in this study) were used in all *in vivo* studies with 5 animals per group. Experiments were conducted without randomization or blinded protocol. Using preliminary peritonitis infection data and a power analysis (using
SPSS for Windows, version 12), a sample size ≥ 2 would be needed to detect a large effect size (d = 0.8) with 95% power using a $t$ test between means with alpha at 0.01. After 1 week of quarantine, inoculation ($t = 0$) was performed by intraperitoneal injection of $300 \mu$L of $2 \times 10^8$ cells, delivered in MEM, of wild-type A. baumannii (ATCC 19606) or MDR A. baumannii (FADDI-AB056) with a 25-gauge syringe. Two groups ($n = 5$ for both ATCC 19606 and FADDI-AB056) received either SNAPP S16 (8.3 mg/kg per dose in MEM, which corresponds to $1.5 \times$ in vitro MBC taking into account the average peritoneal/blood volume of mice) or imipenem (derived from the carbapenem antibiotic family and considered to be the most successful class of antibiotics in evading emerging antimicrobial resistance [4], 40 mg/kg per dose in MEM) treatment 0.5, 4 and 8 h after introduction of the inoculums. An untreated control group was included. Signs of animal distress were monitored, and mice that did not meet distress-related euthanasia criteria at $t < 24$ h were defined as ‘survived’. At $t = 24$ h, all mice were euthanized. Peritoneal washes were performed by injecting 3.0 mL of sterile MEM in the intraperitoneal cavity followed by a massage of the abdomen. Subsequently, the abdomen was opened and 3.0 mL of peritoneal fluid was recovered from the peritoneum for analysis of CFU/mL. Spleen of each mouse was removed and suspended in 5.0 mL MEM in a gentleMACS tube which was then subjected to automatic dissociation (gentleMACS dissociator, Miltenyl Biotec). The peritoneal fluid and supernatant from the dissociation of spleen were serially diluted in saline. A 10-µL portion of each dilution was plated on horse blood agar plates and incubated overnight at 37 °C. For mice that were still alive directly before euthanization, blood was also taken from the heart for immediate plating on horse blood agar plates. Colonies were counted and expressed as CFU/mL, and viable bacteria cell counts in the peritoneal cavity, blood and spleen were compared with those of the control group at 24 h.. The bacterial levels were statistically analyzed using a one-way classification ANOVA and student’s $t$-test (SPSS for Windows, version 12). Data is expressed as mean ± standard deviation (SD) of five biological replicates.

**Fluorescent tagging of SNAPP S16 with Alexa Fluor 488.** SNAPP S16 was dissolved in sodium bicarbonate buffer (0.1 M, pH 8.3) (2.5 mg/mL), and Alexa Fluor 488 (AF488) carboxylic acid succinimidyl ester dissolved in DMSO (10 mg/mL) was added (20 µL/mg of peptide polymer) The mixture was stirred for 1 h at room temperature and then passed through a gel separation column (PD MidiTrap G-25) to remove the excess dye. The filtrate was lyophilized to afford the fluorescently tagged derivative, AF488-S16.
Sample preparation for imaging with 3D-SIM Super-Resolution Microscopy. Sterilized chambered coverglasses were coated with poly-D-lysine (0.1 mL per well from a 0.1 mg/mL stock solution in DPBS) for 90 min. The excess poly-D-lysine was removed by washing with sterilized MilliQ water (2 × 0.5 mL) and the coverglasses were left to dry overnight in a sterile environment. *E. coli* cells (1.25 × 10⁶ cells/mL, prepared as per described in the measurement of MBC) were incubated with AF488-S16 (8 to 256 µg/mL) in a 96-well plate at 37 °C for 90 min. The cell suspension was then transferred to Eppendorf tubes (2 mL) and washed with HBSS twice (5000 g, 10 min). FM® 4-64FX dye (0.2 mL from a 5 µg/mL stock solution in HBSS) was added to the cell pellet and the cells were incubated on ice for 10 min with regular mixing. After incubation, the cells were washed with HBSS (5000 g, 10 min), resuspended in HBSS (0.5 mL), and then transferred to the chambered coverglass. Subsequently, the cells were washed in HBSS (800 g, 10 min) then fixed in 2% w/v paraformaldehyde in PBS for 10 min at room temperature. The fixative was removed with HBSS (800 g, 10 min) and HBSS (0.5 mL) was added to each well prior to imaging.

**LPS inhibition assay.** SNAPP S16 (50 µL) was incubated with LPS from *E. coli O111:B4* (50 µL) in MEM in a 96-well plate at 37 °C for 1 h. *E. coli* cells (which gave an optical density reading of ~0.7 at 650 nm) were diluted to 2.5 × 10⁶ cells/mL in MEM and 100 µL of the bacteria solution was added to the S16-LPS mixture. The final concentration of S16 was kept at 4 µg/mL, whereas the LPS concentration was varied from 2 to 1000 µg/mL. The 96-well plate was then incubated at 37 °C for 90 min. A 50 µL aliquot was taken from each well, transferred to a second 96-well plate and 100 µL of saline and dye mixture (*i.e.*, saline with 0.1% of SYTO® 9 and 0.1% of PI) was added. Each well in the second 96-well plate was analyzed with a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter) to determine the % PI-positive cells. Two independent runs of the assay were conducted and two replicates were used in each run for each variation.

**Kinetics of antimicrobial activity.** SNAPP S16 (at a final concentration of 8 µg/mL) was incubated with *E. coli* cells (at a final concentration of 1.25 × 10⁶ cells/mL) in MEM at 37 °C. Aliquots were taken at t = 0, 15, 30 and 90 min for analysis to determine CFU/mL (refer to procedure for the measurement of MBC) and % of PI-positive cells (*via* flow cytometry using a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter)). An untreated control group was also included. Two independent experiments of the assay were conducted and two replicates were used in each experiment for each variation.
Preparation of Large Unilamellar Vesicles (LUVs) for dye release (pore formation) and lucigenin (chloride ion transport) based assays. To represent a model of an *E. coli* cytoplasmic membrane large unilamellar vesicles (LUVs) consisting of 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) at a 7:3 mole ratio [5], were used to encapsulate 2 mM rhodamine dextran (70 kDa; RD-40) and 2 mM fluorescein dextran (4 kDa; FD-4) in 10 mM Tris and 5 mM NaCl buffer solution (pH 7.3) for the dye release assay or 2 mM lucigenin solution containing NaCl (100 mM), and sodium phosphate salt (10 mM, pH 7.3) for the chloride ion transport assay, using the LUV preparation method we have previously described [6]. The dye release assay was performed as previously described [5] and the chloride ion transport assay conducted as described by Elie *et al.* [7] LUVs were incubated (0.5 h for the dye release experiment or overnight for the chloride ion transport assay) with SNAPP S16 or control AMP maculatin 1.1 at lipid to peptide molar ratios ranging from 50:1 to 10000:1. To afford complete dye release or chloride ion transport, control LUVs were treated with 0.5% v/v Triton X-100. All measurements were made with a Varian Cary Eclipse spectrophotometer (Melbourne, Australia) using a 4-mm path-length quartz microfluorimeter cell (Starna, Hainault, United Kingdom) for the dye release experiment or a FLUOstar Optima plate reader (BMG Labtech, USA) for the chloride ion transport assay. Dye release or chloride ion transport was presented as the percent of fluorescence of RD-70 and FD-4 or lucigenin, respectively, compared to the Triton X-100 control. Data is representative of two independent assays completed in duplicates.

**Membrane potential assay.** Membrane potential was determined by flow cytometry using a BacLight Bacterial Membrane Potential Kit (Invitrogen). When at low concentrations, the dye DiOC$_2$(3) exhibits green fluorescence in all bacterial cells. The fluorescence shifts towards red emission as the dye molecules become more concentrated and self-associate in healthy cells that are maintaining a membrane potential. *E. coli* was inoculated to mid-log phase. Viable cells were then diluted to $2.5 \times 10^6$ cells/mL in PBS and added with variable concentrations (0.5 ×, 1 × and 2 × MBC) of SNAPP S16. A fully depolarized control was provided by the addition of the proton ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at a final concentration of 5 mM to the untreated cells. Prior to a 1 h incubation at 37 °C, 30 mM DiOC$_2$(3) was added to all of the samples. Membrane potential was determined by a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter) as a ratio of cells that exhibited a red fluorescence (FL-3) to those that displayed a green fluorescence...
(FL-1). Gates were drawn based on the untreated (polarized) and CCCP-treated (fully depolarized) controls. Data is representative of two independent assays completed in duplicates.

**RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) analysis for programmed cell death pathways.** To determine if the star peptide polymers induced PCD, *E. coli* cells were incubated with S16 at 1 × and 5 × the MBC, after which the mRNA levels of *recA*, *lexA* and *mazEF* relative to control genes and untreated *E. coli* were determined by RT-PCR [8-10]. *Escherichia coli* ATCC 25922 was grown overnight at 37 °C in LB broth and used to freshly inoculate LB broth (2% v/v inoculum) and was grown to mid-log phase (O.D._600 = 0.6) at 37 °C. A 200 µL aliquot of the cell suspension was mixed with 1 µL of Syto9 and 1 µL of PI and counted on the Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter). After counting, cells were collected by centrifugation at 8000 g for 10 min at 4 °C, washed twice in MEM and finally resuspended at 2 × 10⁶ cells/mL in MEM. A 500 µL aliquot of a stock solution of SNAPP S16 (final concentration of 1 × MBC or 5 × MBC) was added to 500 µL (final concentration of 1 × 10⁶ cells/mL) of *E. coli* cells. Following incubation (4 h), bacterial cells were collected by centrifugation at 8000 g for 10 min at 4 °C and immediately resuspended in 1 mL RNAProtection Bacteria Reagent (Qiagen).

Total RNA was extracted using the RNAProtection bacterial reagent and RNeasy kit (Qiagen). Cells were collected by centrifugation at 8000 g for 10 min at 4 °C and resuspended in 100 µL TE buffer (10 mM TrisCl, 1 mM EDTA, pH 8.0) containing 1 mg/ml lysozyme. After a 5 min incubation at room temperature, 350 µL of buffer RLT was added and the solution mixed by vortexing. Ethanol (250 µL) was then added and the entire 700 µL was added onto an RNeasy spin column and centrifuged at 16000 g for 1 min. The membrane was washed with 700 µL of buffer RW1 followed by 500 µL of buffer RPE. A final centrifugation at 16000 g for 1 min was performed to dry the membrane. RNase free water (50 µL) was added to the membrane and the RNA was eluted by centrifugation at 16000 g for 1 min. RNA was quantified by absorbance (260 nm/280 nm) using a Nanodrop spectrophotometer (Thermo Scientific).

Extracted RNA was immediately DNase-treated using the TURBO DNA-free kit (Ambion). Briefly, 5 µg of RNA was combined with 2 µL of 10× DNase buffer, 1 µL of DNase and sufficient water to make up a 20 µL reaction volume. The reaction was incubated for 20 min at 37 °C, after which a further 1 µL of DNase was added and the reaction
incubated for another 20 min at 37 °C. Following this second incubation, 2 µL of DNase inactivation reagent was added. After a 5 min incubation at room temperature with occasional mixing, the inactivation reagent was pelleted by centrifugation at 10000 g for 1 min and the supernatant collected.

Reverse transcription was performed using the iScript Reverse Transcription Supermix (Bio-Rad). Briefly, 1 µg (4 µL) of the DNase-treated RNA extract was combined with 4 µL of iScript master mix and 12 µL of RNase free water. The reverse transcription reaction was performed with a 5 min, 25 °C priming step, a 30 min, 42 °C extension step followed by a 5 min, 85 °C inactivation step. A no-reverse transcription reaction was also set up using 1 µg (4 µL) of the DNase treated RNA extraction combined with 16 µL of RNase free water.

RT-PCR was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad). Template cDNA (25 ng, 1 µL) was combined with 0.8 µL of forward primer (5 nM), 0.8 µL of reverse primer (5 nM) and 10 µL of Sybr Green Supermix. Primers used for the RT-PCR; recA (For) AGATCCTCTACGGCGAAGGT, (rev) CCTGCTTTTCTCGATCAGCTT; lexA (For) GACTTGCTGGCAGTGCATAA, (rev) TCAGGCGCTTAACGGTAACT; MazEF-1 (For) CTTCCGTGCTCCTCTTGC, (rev) CGTGGGGAAATTCACCG; 16SrRNA (For) TGTAGCGGTGAAATGCGTAGA, (rev) CACCTGAGCGTCAGTCTTCGT [9]. Thermal cycling was performed using 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. A positive control of genomic DNA and a no-reverse transcription control were included in each cycling run. Cycling was performed on a Rotor Gene RG-3000A Thermal Cycler running Rotor-Gene V6.1 software (Corbett Research). Analysis of the PCR was performed using the LinRegPCR software version 2015.3. Comparative cycle threshold (Ct) analysis was performed according to the method of Schmittgen and Livak [11].

**ROS production.** *E. coli* cells (which gave an optical density reading of ∼0.7 at 650 nm) were diluted to 2.5 × 10^6 cells/mL in MEM and 100 µL of the bacteria solution was added to each well containing either MEM (untreated control) or the test compound(s) at the desired concentrations (100 µL). The 96-well plate was then incubated at 37 °C for 90 min. The cells were then stained with the CellROX® Orange Reagent at a final concentration of 750 nM following manufacturer’s instructions and were incubated for 1 h at 37 °C. The cells were analyzed on the Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter) where the fluorescence from the CellROX® Orange Reagent was measured on FL-3. A minimum of two
independent experiments of the assay were conducted and two technical replicates were used in each experiment. Data is expressed as mean ± standard deviation.

Effect of ALD inhibition on the membrane disruption ability of SNAPP S16. *E. coli* cells (which gave an optical density reading of ∼0.7 at 650 nm) were diluted to 2.5 × 10^6 cells/mL in MHB, and 5 mL of the bacterial cell solution was added to an equimolar of doxycycline hyclate (to yield a final concentration equivalent to its MIC of 0.5 µg/mL). The mixture was incubated at 37 °C for 4 h and the cells were recovered via centrifugation (3000 g, 10 min) at the end of the incubation period. The recovered cells (100 µL/well) were then incubated at 37 °C for a further 90 min in the absence or presence of SNAPP (at 0.5 × and 1 × MBC, 100 µL/well) in a 96-well plate. A 50 µL aliquot was taken from each well, transferred to a second 96-well plate and 100 µL of saline and dye mixture (*i.e.*, saline with 0.1% of SYTO® 9 and 0.1% of PI) was added. Each well in the second 96-well plate was analyzed with a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter) to determine the % PI-positive cells. Two independent runs of the assay were conducted and two replicates were used in each run for each variation.

### 7.6 Specific Experimental Procedures for Chapter 3

**Synthesis of poly(DL-valine-b-Z-L-lysine)_{arm}PAMAM-(NH₂)_{16,core} star polymers** SB₁₆,Z. Lys NCA (1.3 g, 4.19 mmol) was dissolved in anhydrous DMF (13 mL) and added via syringe to PAMAM-(NH₂)₁₆ (dried, 43 mg, 13.1 µmol) dissolved in anhydrous DMF (1 mL). After stirring for 24 h under argon, Val NCA (0.3 g, 2.1 mmol) dissolved in anhydrous DMF (3 mL) was added to the reaction mixture. The reaction mixture was stirred for a further 24 h under argon, after which *n*-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated polymer solution into diethyl ether (3 × 40 mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded (PVal-b-PZLL)_{arm}PAMAM-(NH₂)₁₆,core star polymer SB₁₆,Z as an off-white solid, 1.2 g (89%).

**Synthesis of poly(DL-valine-b-Z-L-lysine)_{arm}PAMAM-(NH₂)₂₃,core star polymers** SB₃₂,Z. Lys NCA (1.3 g, 4.19 mmol) was dissolved in anhydrous DMF (13 mL) and added via syringe to PAMAM-(NH₂)₃₂ (dried, 45 mg, 6.5 µmol) dissolved in anhydrous DMF (1 mL). After stirring for 24 h under argon, Val NCA (0.3 g, 2.1 mmol) dissolved in anhydrous DMF (3 mL) was added to the reaction mixture. The reaction mixture was stirred for a further 24 h under argon, after which *n*-butyl alcohol (1 mL) was added and the mixture was stirred
for a further 1 h. Precipitation of the concentrated polymer solution into diethyl ether (3 × 40 mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded (PVal-b-PZLL)\textsubscript{arm}PAMAM-(NH\textsubscript{2})\textsubscript{32,core} star polymer \textbf{SB\textsubscript{32,Z}} as an off-white solid, 1.17 g (87%).

**Synthesis of poly(Z-L-lysine-\textit{r}-DL-valine)\textsubscript{arm}PAMAM-(NH\textsubscript{2})\textsubscript{16,core} star polymers \textbf{SR\textsubscript{16,Z}}.** Lys NCA (1.3 g, 4.19 mmol) and Val NCA (0.3 g, 2.1 mmol) were dissolved in anhydrous DMF (16 mL) and added via syringe to PAMAM-(NH\textsubscript{2})\textsubscript{16} (dried, 43 mg, 13.1 µmol) dissolved in anhydrous DMF (1 mL). After stirring for 24 h under argon, \textit{n}-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated polymer solution into diethyl ether (3 × 40 mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded (PZLL-\textit{r}-PVal)\textsubscript{arm}PAMAM-(NH\textsubscript{2})\textsubscript{16,core} star polymer \textbf{SR\textsubscript{16,Z}} as an off-white solid, 1.21 g (90%).

**Synthesis of poly(Z-L-lysine-\textit{r}-DL-valine)\textsubscript{arm}PAMAM-(NH\textsubscript{2})\textsubscript{32,core} star polymers \textbf{SR\textsubscript{32,Z}}.** Lys NCA (1.3 g, 4.19 mmol) and Val NCA (0.3 g, 2.1 mmol) were dissolved in anhydrous DMF (16 mL) and added via syringe to PAMAM-(NH\textsubscript{2})\textsubscript{32} (dried, 43 mg, 13.1 µmol) dissolved in anhydrous DMF (1 mL). After stirring for 24 h under argon, \textit{n}-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated polymer solution into diethyl ether (3 × 40 mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded (PZLL-\textit{r}-PVal)\textsubscript{arm}PAMAM-(NH\textsubscript{2})\textsubscript{32,core} star polymer \textbf{SR\textsubscript{32,Z}} as an off-white solid, 1.15 g (85%).

**Synthesis of poly(Z-L-lysine)\textsubscript{arm}PAMAM-(NH\textsubscript{2})\textsubscript{16,core} star polymer \textbf{SH\textsubscript{16,Z}}.** Lys NCA (1 g, 3.26 mmol) was dissolved in anhydrous DMF (9 mL) and added via syringe to PAMAM-(NH\textsubscript{2})\textsubscript{16} (dried, 33 mg, 10.2 µmol) dissolved in anhydrous DMF (1 mL). After stirring for 24 h under argon, \textit{n}-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated polymer solution into diethyl ether (3 × 40 mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded PZLL\textsubscript{arm}PAMAM-(NH\textsubscript{2})\textsubscript{16,core} star polymer \textbf{SH\textsubscript{16,Z}} as an off-white solid, 770 mg (87%).

**Synthesis of poly(Z-L-lysine)\textsubscript{arm}PAMAM-(NH\textsubscript{2})\textsubscript{32,core} star polymer \textbf{SH\textsubscript{32,Z}}.** Lys NCA (1 g, 3.26 mmol) was dissolved in anhydrous DMF (9 mL) and added via syringe to PAMAM-(NH\textsubscript{2})\textsubscript{32} (dried, 35 mg, 5.1 µmol) dissolved in anhydrous DMF (1 mL). After stirring for 24 h under argon, \textit{n}-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated polymer solution into diethyl ether (3 × 40
mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded PZLL-armPAMAM-(NH\textsubscript{2})\textsubscript{32},core star polymer SH\textsubscript{32,Z} as an off-white solid, 780 mg (87%).

**Synthesis of linear poly(Z-L-lysine) polymer LH\textsubscript{Z}**. Lys NCA (0.5 g, 1.63 mmol) was dissolved in anhydrous DMF (5 mL) and added via syringe to 4-methylbenzylamine (6.9 µL, 54.4 µmol). After stirring for 24 h under argon, n-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated polymer solution into diethyl ether (3 × 40 mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded linear PZLL polymer LH\textsubscript{Z} as an off-white solid, 411 mg (95%).

**Synthesis of linear poly(Z-L-lysine-r-DL-valine) polymer LR\textsubscript{Z}**. Lys NCA (0.5 g, 1.63 mmol) and Val NCA (117 mg, 0.82 mmol) were dissolved in anhydrous DMF (6 mL) and added via syringe to benzylamine (10.3 µL, 81.6 µmol). After stirring for 24 h under argon, n-butyl alcohol (1 mL) was added and the mixture was stirred for a further 1 h. Precipitation of the concentrated polymer solution into diethyl ether (3 × 40 mL), followed by isolation via centrifugation and drying (0.1 mbar), afforded linear PZLL-r-PVal polymer LR\textsubscript{Z} as an off-white solid, 420 mg (81%).

### 7.7 Specific Experimental Procedures for Chapter 4

**NPN uptake (Outer membrane, OM, permeability) assays.** The assays were conducted in either mSBF or SBF. A dilution series of SNAPP S\textsubscript{16} was made by diluting S\textsubscript{16} stock in medium to concentrations of 0.2 to 11.7 µM and a final volume of 50 µL in each well of a 96-well plate. A 5 mM NPN stock solution in acetone was prepared which was then diluted to 40 µM in the medium of interest before use. S\textsubscript{16} (50 µL), NPN (40 µM, 50 µL), and medium (50 µL) were pipetted into the 96-well plates in advance, and either *E. coli* or *A. baumannii* cell suspension (50 µL, 5 × 10\textsuperscript{6} cells/mL in medium) was added to each well immediately before fluorescence measurement using a microplate reader (PerkinElmer 1420 Multilabel Counter VICTOR\textsuperscript{3}). The fluorescence values were recorded after 5 min and until a plateau was reached. Control wells which included (i) medium alone (200 µL), (ii) medium (150 µL) and bacterial cell suspension (50 µL), (iii) medium (150 µL) and NPN (40 µM, 50 µL), and (iv) medium (100 µL), NPN (40 µM, 50 µL) and bacterial cell suspension (50 µL) were incorporated. For *E. coli*, an additional set was conducted in SBF with EDTA (final concentration of 1.5 mg/mL EDTA) as an additive. The results are expressed in relative fluorescence units (RFUs) where the fluorescence value of cell suspension and NPN (control
iv) was subtracted from that of the test well RFUs. A minimum of two independent experiments (biological replicates) of the assay were conducted and two technical replicates were used in each experiment for each bacterial species and S16 concentration. Data is expressed as mean ± standard deviation (SD) of the biological replicates.

**Cytoplasmic membrane (CM) disruption assays using flow cytometry.** Briefly, the CM disruption assay was conducted as previously described. Bacterial cells (100 µL, final concentration of 2.5 × 10⁶ cells/mL) were added to SNAPP S16 (100 µL, final concentration of 0.09 µM) in the relevant medium in a 96-well plate. The plate was then incubated at 37 °C for 90 min. A 50 µL aliquot was taken from each well, transferred to a second 96-well plate and 100 µL of saline and dye mixture (i.e., saline with 0.1% of SYTO® 9 and 0.1% of PI) was added. Each well in the second 96-well plate was analyzed with a Cell Lab Quanta SC MPL flow cytometer to determine the % of cells with intact membranes (PI-negative) and cells with compromised membranes (PI-positive). Positive controls containing cells alone were incorporated. Two independent runs of the assay were conducted and two replicates were used in each run for each bacteria.

**Investigating the effect of EDTA on the MIC of SNAPP S16.** The assays were conducted using the broth microdilution method described above, except that different concentrations of EDTA disodium salt (0.5 to 2 mg/mL) were added to the S16 solution prior to the addition of bacterial cells.
7.8 Specific Experimental Procedures for Chapter 5

Quantifying synergy using the Bliss Independence and Highest Single Agent (HSA) models. The Bliss Independence and HSA models were used to calculate drug synergism [13,14]. With the Bliss Independence model, synergism was calculated using the formula, $BIC_{AB} = A + B - AB$ (1), where A and B are the effects of the two drugs in isolation and $BIC_{AB}$ is the combined effect of the two drugs as predicted by the Bliss Independence model. Synergy is observed if $C_{AB}$, which is the experimentally determined combined effect of the two drugs, is greater than $BIC_{AB}$. In the HSA model, if $C_{AB}$ is greater than the greater of A and B, $C_{AB} > \max(A, B)$, synergy is observed. The quantitative effect of the compounds when administered alone (i.e., A and B) or in combination (i.e., $C_{AB}$) was defined as the fractional reduction of the population, $R = 1 - \frac{CFU_t}{CFU_0}$, where $CFU_t$ is the CFU/mL measured after treatment, and $CFU_0$ is the CFU/mL measured before treatment.

7.9 References


APPENDIX I

Supplementary Information for Chapter 2

Synthesis and Characterization of SNAPPs

The synthetic scheme of SNAPPs is provided in Supplementary Figure 2.1. Successful synthesis of SNAPPs was confirmed by $^1$H NMR spectroscopic analysis (Supplementary Figures 2.3a and 2.4a), which also allowed calculation of the actual lysine-to-valine ratios to be $\sim 2:1$ for both $\text{S}16_Z$ and $\text{S}32_Z$ (Supplementary Figure 2.5 and Supplementary Table 2.1). The subsequent removal of the carboxylbenzyl (Cbz) protecting groups on the lysine residues of SNAPPs yielded water-soluble stars $\text{S}16$ and $\text{S}32$ with pendent primary amine functionalities along the star arms (Figure 2.1; see Supplementary Figures 2.3b and 2.4b for $^1$H NMR spectroscopic analysis), which would remain protonated at physiological pH (pKa = 10.5). The molecular weight characteristics of SNAPPs were estimated by aqueous gel permeation chromatography (GPC) (number-average molecular weight ($M_n$) = 43.8 kDa for $\text{S}16$ and 74.8 kDa for $\text{S}32$) (Supplementary Table 2.1). The solvated dimensions of SNAPPs $\text{S}16$ and $\text{S}32$ were studied via dynamic light scattering (DLS) analysis in minimal essential medium (MEM), which revealed monomodal particle size distributions and hydrodynamic diameters ($D_H$) of 7.7 and 13.5 nm, respectively (Supplementary Figures 2.6 and 2.7). The sizes of SNAPPs were further substantiated by transmission electron microscopy (TEM) analysis which showed that $\text{S}16$ and $\text{S}32$ have uniform diameters of 7.8 ± 1.2 and 7.5 ± 1.6 nm, respectively (Supplementary Figure 2.8).
Supplementary Figure 2.1. Synthesis of SNAPPs. Synthesis of SNAPPs via ROP of lysine and valine NCAs was initiated from the terminal amines of PAMAM dendrimers. Second (G2) and third (G3) generation PAMAM dendrimers (see Supplementary Figure 2.2 for structure of the former) with 16 and 32 peripheral primary amines were used to prepare 16- and 32-arm SNAPPs, respectively. The number of repeat units for lysine and valine are $a$ and $b$, respectively. The lysine-to-valine ratios (i.e., $a:b$) are provided in Supplementary Table 2.1.
Supplementary Figure 2.2. Structure of a second generation PAMAM dendrimer (G0 = generation 0; G1 = generation 1; G2 = generation 2).
1H NMR Spectroscopic Analysis of SNAPPs

1H NMR spectroscopic analysis (Supplementary Figures 2.3 and 2.4) was employed to confirm successful synthesis of SNAPPs. Proton resonances characteristic of valine (i.e., $\delta_\text{H} 0.8$ ppm corresponding to the methyl groups on the valine side chain) and lysine (i.e., $\delta_\text{H} 1.2$-$1.8$ ppm corresponding to the methylene protons on the lysine side chain) residues were observed. Integration and comparison of these resonances (Supplementary Figure 2.5) provided lysine-to-valine ratios of approximately 2:1, which is consistent with the ratio of lysine and valine NCA monomers used in the synthesis (Supplementary Table 2.1). Resonances resulting from the G2 and G3 PAMAM cores were difficult to observe upon star formation as they overlap with the broad peptide polymer peaks [1]. It is also likely that the intensities of the core resonances are much smaller relative to those resulting from the star arms as the contribution of the PAMAM core to the overall molecular weight of the star is relatively small.
Supplementary Figure 2.3. a-b, $^1$H NMR spectra ($d_6$-DMSO) of 16-arm Cbz-protected star peptide polymer S16$^Z$ (a) and deprotected star peptide polymer (SNAPP) S16 (b). The spectra are representative of four independent experiments.
Supplementary Figure 2.4. a-b, $^1$H NMR spectra ($d_6$-DMSO) of 32-arm Cbz-protected star peptide polymer S32$_Z$ (a) and deprotected star peptide polymer (SNAPP) S32 (b). The spectra are representative of four independent experiments.
Example of Integration of $^1$H NMR Spectra

Supplementary Figure 2.5. Integration of $^1$H NMR spectrum ($d_6$-DMSO) of star peptide polymer S16Z to determine the lysine-to-valine ratio.
Size Analysis of SNAPPs

**Supplementary Figure 2.6.** DLS normalized mass % of SNAPPs as a function of hydrodynamic diameter ($D_H$). The numbers on the DLS distributions show the average $D_H$ of SNAPPs in minimal essential medium (MEM; red dotted lines) and Mueller-Hinton broth (MHB; green dashed lines). The DLS distributions for the ‘blank’ MEM and MHB are also shown. For each sample, the average $D_H$ was determined as an average of 3 sets containing 15 measurements per set. The data are representative of three independent experiments.
Supplementary Figure 2.7. Intensity autocorrelation curves corresponding to the DLS traces of S16 and S32 in minimal essential medium (MEM) and Mueller-Hinton broth (MHB). The autocorrelation curve that corresponds to the DLS distribution of ‘blank’ MHB is also provided. The data are representative of three independent experiments.
Supplementary Figure 2.8. a-b. TEM analysis of SNAPPs S16 (a) and S32 (b) in minimal essential media (MEM). The samples were negative-stained and air dried at a sample concentration of 0.5 µg/mL. The images are representative of three independent experiments.

**S16**
Diameter = 7.8 ± 1.2 nm

**S32**
Diameter = 7.5 ± 1.6 nm
Supplementary Figure 2.9. Method for MBC determination. a-b, Sample concentration-killing curves and linear regression analysis used to determine MBC values. Error bars represent the standard deviation from the mean ($n = 4$). MBC was determined as the concentration at which colony-forming unit (CFU)/mL becomes zero. The examples shown were based on experimental data obtained for S16 against *P. aeruginosa* in Mueller-Hinton broth (MHB) (a) and S32 against multidrug resistant (MDR) *A. baumannii* in minimal essential medium (MEM) (b). The MBC values calculated were 1.42 µM for (a) and 0.03 µM for (b).
Method used for calculating MBCs (molar concentrations):
The number average molecular weight, $M_n$, obtained from GPC analysis was consistently used in this study to convert mass concentrations to molar concentrations. The reader is referred to Supplementary Table 2.1 and Supplementary Table 2.7 for the $M_n$ of SNAPPs and their linear analog, respectively.

The following formula was used:

$$\text{Molar concentration (µM)} = \frac{\text{Mass concentration (µg/mL)}}{M_n \text{ (g/mol)}} \times 1000$$

The calculation is exemplified as below:

MBC of S16 against E. coli in MEM = 7.6 µg/mL 
$M_n$ of S16 = 43800 g/mol 
Therefore,

$$\text{MBC (µM)} = \frac{7.6 \text{ µg/mL}}{43800 \text{ g/mol}} \times 1000 = 0.17 \text{ µM}$$
Resistance Study

Supplementary Figure 2.10. Resistance acquisition in the presence of sub-MBC levels of star S16. Wild type (ATCC 19606) and colistin-resistant MDR (FADDI-AB156) A. baumannii were serially passaged in MHB containing sub-MBC levels of star peptide polymer S16. The change in the MBC of star peptide polymer S16 against both strains of A. baumannii is shown on the y axis over 600 generations of growth. Note that MBC determination was conducted in triplicates completed in two independent experiments.
Synthesis and Characterization of the Linear Random Co-peptide Polymer

Supplementary Figure 2.11. Synthesis of linear peptide polymer \( \text{L}_z \). The ROP of lysine NCA and valine NCA initiated by 4-methylbenzylamine, followed by deprotection of the carboxybenzyl groups on the lysine residues with HBr and subsequent dialysis in RO water to afford water-soluble linear peptide polymer \( \text{L} \). The number of repeating units for lysine and valine are \( a \) and \( b \), respectively. The lysine-to-valine ratio, \( a:b \), is provided in Supplementary Table 2.7.
Supplementary Figure 2.12. a-b, ^1^H NMR spectra (d_6-DMSO) of linear Cbz-protected peptide polymer L, (a) and deprotected peptide polymer L (b). The spectra are representative of three independent experiments.

Note on ^1^H NMR Spectra of the Linear Random Co-peptide Polymer:

For linear random co-peptide polymer L, the peaks associated with the 4-methylbenzylamine initiator could be observed, which enabled the number-averaged degree of peptide polymerisation (DP_n) to be determined. The DP_n value for L was found to be 36 repeat units, which is close to the targeted value of 30.
Supplementary Figure 2.13. DLS and TEM analysis of the linear random co-peptide polymer \( L \). a-b, DLS normalized mass % of the linear random co-peptide polymer \( L \) in minimal essential medium (MEM) (a) as a function of hydrodynamic diameter \( D_H \), where the average \( D_H \) was determined as an average of 3 sets of 15 measurements per set. The intensity autocorrelation curve corresponding to the DLS trace is shown in (b). c, TEM analysis of \( L \) (negative-staining, air dried, sample concentration of 0.5 µg/mL). The data in a and b and the image in c are representative of three independent experiments.
Cryo-TEM Images of SNAPP S16 in MHB

Supplementary Figure 2.14. Visualization of SNAPP in MHB using cryo-TEM. a-d, Cryo-TEM images of S16 in MHB at 35 µg/mL (0.8 µM). All images are representative of three independent experiments.
DLS Analysis of SNAPP S16 in Fetal Bovine Serum (FBS)

Supplementary Figure 2.15. DLS analysis of SNAPP S16 in 1% fetal bovine serum (FBS). DLS normalized mass % of 1% FBS with or without SNAPP S16 as a function of hydrodynamic diameter ($D_H$) was determined as an average of 3 sets of 15 measurements per set. The intensity autocorrelation curves corresponding to the DLS traces are shown. The numbers on the DLS distributions show the average $D_H$ of each peak. For the sample containing S16 in 1% FBS, a multimodal distribution was obtained and the % mass contributing to each peak was listed in parentheses.

Note:
The addition of S16 to 1% FBS resulted in the detection of larger sized populations not present in pure 1% FBS. These populations are attributed to the formation of protein-S16 aggregates, with the majority possessing an average $D_H$ of 125.1 nm. The peak at 6.6 nm is likely contributed by proteins found in FBS and possibly non-aggregated S16 particles.
Results from Hemolysis Assays

Supplementary Figure 2.16. Percent hemolysis as a function of SNAPP concentration. Error bars represent the standard deviation from the mean ($n = 4$).
**Supplementary In Vivo Data**

**Supplementary Figure 2.17. In vivo efficacy of SNAPP S16 in a mouse peritonitis (A. baumannii ATCC 19606-infected) model.** a-b. Colony forming units (CFU) of A. baumannii (ATCC 19606) found in the blood (a) and spleen (b) of infected mice 24 h after no treatment or treatment with imipenem (40 mg/kg) or S16 (8.3 mg/kg). All data are expressed as mean ± standard deviation as indicated by the error bars, based on values obtained from five biological replicates (n = 5). *P<0.01, Student’s t test, significant difference from the untreated control.
Supplementary Figure 2.18. *In vivo* efficacy of SNAPP S16 in a mouse peritonitis (MDR *A. baumannii* FADDI-AB156-infected) model. a-b. Colony forming units (CFU) of MDR *A. baumannii* (FADDI-AB156) found in the blood (a) and spleen (b) of infected mice 24 h after no treatment or treatment with imipenem (40 mg/kg) or S16 (8.3 mg/kg). All data are expressed as mean ± standard deviation as indicated by the error bars, based on values obtained from four biological replicates (*n* = 4). *P* < 0.01, **P** < 0.001, Student’s *t* test, significant difference from the untreated control.
Conjugation of AF488 to S16

Determining the degree of labelling (DOL):

A calibration curve (Supplementary Figure 2.19) was constructed to estimate the degree of AF488 conjugation on SNAPP S16. Note that the DOL is calculated based on the assumption that the absorbances of the free and conjugated dye are the same (which might not be the case). In this study, an estimated DOL is sufficient.

The labelled SNAPP (5.2 mg/mL) was found to have an absorbance of 0.78 at 490 nm. This correlates to approximately 0.11 mg/mL of AF488 dye based on the calibration curve. Taking into the account the molarities of the dye and SNAPP in solution, the DOL was found to be approximately 3 dye molecules per SNAPP S16 molecule.

Supplementary Figure 2.19. The calibration curve used to estimate the amount of AF488 dye conjugated based on the absorbance at 490 nm. Sterile water was used to dissolve the AF488 dye. A linear trend line (red) was fitted over the acquired data points (slope = 4.333, y-intercept = 0.297, $R^2 = 0.96$). The data are representative of two independent experiments.
**Supplementary Figure 2.20.** OMX 3D-SIM images of *E. coli* before and after treatment with AF488-tagged SNAPP S16 in MHB. 

**a.** Image of untreated *E. coli*. 

**b-e.** Images of *E. coli* incubated with AF488-tagged SNAPP S16 at 2 × MBCtagged. The *E. coli* cell membrane was stained with FM4-64FX (red) and the star peptide polymer with Alexa Fluor 488 (green) in all images. Note that the MBC used refers to the MBC of the fluorescently tagged star (Supplementary Table 2.11). All images are representative of three independent experiments.
Supplementary Figure 2.21. OMX 3D-SIM images of *E. coli* after treatment with AF488-tagged SNAPP S16 at \( 1 \times \) MBC-tagged in MHB. **a-b.** Examples of the orthogonal projections of Figure 2.3c (**a**) and Figure 2.3e (**b**) on the xy-, xz- and yz-planes. The *E. coli* cell membrane was stained with FM4-64FX (red) and S16 with AF488 (green) in all images. All images are representative of three independent experiments.
Supplementary Figure 2.22. OMX 3D-SIM images of *E. coli* after treatment with AF488-tagged SNAPP S16 at approximately 2 × MBC in MEM. *a-d*, Z-projection images. *e*, An example of the orthogonal projections of image *b* on the xy-, xz- and yz-planes. *f*, An example of the orthogonal projections of image *c* on the xy-, xz- and yz-planes. The *E. coli* cell membrane was stained with FM4-64FX (red) and S16 with AF488 (green) in all images. All images are representative of three independent experiments.
Results from LPS Inhibition Assays

Supplementary Figure 2.23. Percentage of PI-positive (membrane-disrupted) E. coli cells as a function of LPS concentration. The concentration of S16 used was fixed at 4 µg/mL (0.09 µM). Error bars represent the standard deviation from the mean (n = 4).
Supplementary Figure 2.24. Flow cytometric analysis of *E. coli* cells in the LPS inhibition assay. a-l. Two-parameter dot plots and histograms obtained from the analysis of samples containing *E. coli* and LPS (a), *E. coli* and S16 (b), and *E. coli*, S16 and varying concentrations of LPS (2 to 1000 µg/mL) (c-l). The concentration of S16 used was fixed at 4 µg/mL (0.09 µM, i.e., MBC<sub>50</sub> in MEM). Samples were incubated at 37 °C for 90 min prior to analysis. On the two-parameter dot plots, the x-axis represents fluorescent channel 1 (FL-1), which measures the fluorescent emission of SYTO<sup>®</sup> 9. The y-axis represents fluorescent channel 2 (FL-2), which measures the fluorescent emission of PI. All data are representative of three independent experiments.
Correlation of Antibacterial Activity with the Cellular Uptake of S16

Flow cytometric analysis was used to correlate the cellular uptake of SNAPP with the resulting bacterial membrane lysis. As a proof of concept, the same amount of the model gram-negative bacteria, *E. coli*, was treated with Alexa Fluor 488-tagged SNAPP **S16** of varying concentrations using MHB as the medium and the mixtures were then subjected to analysis using the Cell Lab Quanta SC MPL system. The propidium iodide (PI) nucleic acid stain was added to the *E. coli*-*S16* mixture to quantify cells with compromised membranes. Utilizing the ability of flow cytometers to provide accurate quantification of cell numbers, the counts of AF488- and PI-positive cells were measured for each **S16** concentration (Supplementary Figure 2.25).

Based on Supplementary Figure 2.25, as the concentration of AF488-tagged **S16** increases, the number of cells with compromised membranes (PI-positive cells) increases as expected and plateaus at about 128 µg/mL (2.92 µM). Similarly, the number of AF488-positive cells follows the same trend. As the cells were not tagged with AF488, the presence of AF488-positive cells correlates with cells which are associated with the AF488-tagged **S16**. It is noteworthy that the gating of the flow cytometer was done so as to exclude populations such as free **S16** with significantly smaller sizes than bacterial cells. Hence, this indicates that **S16** association with the cells increases with **S16** concentration and plateaus at high concentrations in a similar fashion as the lysis of bacterial cells. Based on the similar trends exhibited for the cellular association of peptide polymer AF488-tagged **S16** and bacterial membrane lysis as **S16** concentration increases, a direct correlation between these two parameters is indicated. By plotting one parameter against the other (Supplementary Figure 2.26), we then conclude that the uptake of AF488-tagged **S16** is proportional to the membrane disruption of *E. coli*. 
Supplementary Figure 2.25. Total counts of PI+ cells and cells associated with AF488-tagged S16 as a function of S16 concentration. Error bars represent the standard deviation from the mean (n = 4).
Supplementary Figure 2.26. Total counts of PI+ cells as a function of the total counts of cells associated with AF488-tagged S16. A linear trend line (red) was fitted over the acquired data points (slope = 4.078, y-intercept = 2010, $R^2 = 0.93$). All assays were conducted in quadruplicates over two independent runs.
Kinetics of Antimicrobial Activity

Supplementary Figure 2.27. Effects of SNAPP-bacteria incubation period on antimicrobial activity; measured in terms of CFU/mL and % PI-positive or membrane-disrupted cells. The concentration of SNAPP S16 used was ~ 1 × MBC (i.e., 8 µg/mL). All data are expressed as mean ± standard deviation as indicated by the error bars (n = 4). *P<0.05, **P<0.001, Student’s t test, significant difference from the untreated control at the corresponding time points.
Results from Large Unilamellar Vesicle Studies

Supplementary Figure 2.28. Large unilamellar vesicle (LUV) studies. a, Effect of SNAPP S16 and maculatin concentration on the release of rhodamine-dextran 70 kDa (RD-70, black bars) and fluorescein-dextran 4 kDa (FD-4, grey bars) from POPE/POPG LUVs. RD-70 and FD-4 loaded LUVs were incubated with SNAPP S16 or control AMP maculatin 1.1 with a lipid/peptide molar ratio of 1000:1 or 100:1 for 0.5 h at 37°C, after which supernatant was removed from lipid pellets. The excitation wavelengths for RD-70 and FD-4 excitations were 550 and 480 nm, respectively. Emissions were recorded from 560 to 650 nm. b, Effect of SNAPP S16 (grey bars) and maculatin 1.1 (black bars) concentrations on chloride ion efflux from POPE/POPG LUVs. LUVs were loaded with lucigenin solution containing NaCl (100 mM), and sodium phosphate salt (10 mM, pH 7.3) and incubated with SNAPP S16 or control AMP maculatin 1.1 in NaNO₃ (100 mM, pH 7.3) with a lipid/peptide molar ratio of 50:1 to 6400:1 for 18 h at 37°C, after which the lucigenin fluorescence was monitored by excitation at 372 nm, and the emission was recorded at 503 nm. All data are expressed as mean ± standard deviation as indicated by the error bars (n = 4).

Note:

Supplementary Figure 2.28a: S16 resulted in only a marginal release of the 4 kDa or 70 kDa dextrans from the LUVs, which was in stark contrast to the release of dextrans induced by the pore-forming AMP, maculatin 1.1.

Supplementary Figure 2.28b: S16 induced a 50% chloride ion efflux (EC₅₀) at a concentration of ca. 2.7 µg/mL (corresponding to a lipid to S16 molar ratio of 4798±431:1) which was similar to the EC₅₀ of maculatin 1.1 (ca. 1.7 µg/mL at a lipid to peptide molar ratio of 381±46:1).
Membrane Potential Analysis

Supplementary Figure 2.29. Membrane potential flow cytometric dot plots obtained after incubating *E. coli* with 30 µM DiOC$_2$(3) for 1 h in the presence/absence of CCCP (a proton ionophore), and SNAPP 16 at 0.5 ×, 1 ×, and 2 × its MBC. The controls where CCCP was either absent (-CCCP) or present (+CCCP) represent the normal membrane potential state and fully depolarized state for *E. coli*, respectively. A flow cytometry gate (black polygon) was drawn in each panel to indicate the position of the viable bacteria in the absence of CCCP or SNAPP.
Supplementary Figure 2.30. Membrane potential measurements in *E. coli* with serial SNAPP addition. Bacterial membrane potential was indicated by the ratio of cells that exhibited a red fluorescence (*i.e.*, healthy or hyperpolarized cells as captured on FL-3) to those that displayed a green fluorescence (*i.e.*, depolarized cells as captured on FL-1). *E. coli* bacterial cells were incubated with 30 µM DiOC$_2$(3) for 1 h in the presence or absence of CCCP, and with 0.5 ×, 1 ×, and 2 × the MBC of SNAPP S16. All data are expressed as mean ± standard deviation as indicated by the error bars (*n* = 4).
**Results from Programmed Cell Death Studies**

Supplementary Figure 2.31. Programmed cell death PCR studies. SNAPP S16 induced programmed cell death in *E. coli* via the apoptotic-like death (ALD, *recA* and *lexA* expression) pathway but not the MazEF pathway. All data were expressed as mean ± standard deviation as indicated by the error bars (*n* = 4) of the fold increase in RNA levels as determined by real-time PCR from untreated cells. *P*<0.05, Student’s *t* test, significant difference from the untreated control.
Supplementary Figure 2.32. Fold change in percentage of *E. coli* cells stained with CellROX® Orange relative to the untreated control. All data are expressed as mean ± standard deviation as indicated by the error bars (*n* = 4). *P*<0.05, Student’s *t* test, significant difference from the untreated control.

SNAPP S16 induces reactive oxygen species (ROS) production

We measured the production of ROS in untreated *E. coli* cells and in cells treated with S16 (at 0.25 ×, 0.5 ×, and 1 × its MBC), thiourea (75 mM) or a combination of S16 (0.5 × MBC) and thiourea (75 mM) for 90 min using the CellROX® Orange Reagent (Supplementary Figure 2.32). The reagent is non-fluorescent in a reduced state and exhibits bright orange fluorescence (*ca.* 545/565 nm) when oxidized by ROS. Hence, the cells were analysed via flow cytometry to determine the percentage of ROS-producing cells as indicated by the amount of cells stained with the reagent (*i.e.*, CellROX® Orange-positive cells). A slight increase (*i.e.*, 2-fold relative to the untreated control) in cells stained with CellROX® Orange was observed when the cells were treated with S16 at 0.25 × MBC. When the S16 concentration was increased to 0.5 × MBC, more than a 6-fold increase in the percentage of
cells stained with CellROX® Orange was observed compared to the untreated control, indicating increased ROS production. The addition of thiourea, a ROS scavenger [2], inhibited ROS production by S16 at 0.5 × MBC as indicated by a reduction in the percentage of cells stained with the reagent (i.e., less than a 1-fold increase compared to the untreated control). We observed that the treatment of E. coli with S16 at its MBC resulted in a negligible increase in ROS production. This could be due to significant and rapid cell lysis and fragmentation upon treatment with S16 at its lethal dose that occur well within the 90 min incubation period, resulting in the inability to sample the cells at the stage where ROS is being produced prior to cell lysis. In support of the postulation, we noted a significant reduction in the number of bacterial cell population (>3-log reduction compared to the untreated control) and a high percentage (> 90%) of membrane-disrupted cells after 90 min treatment with S16 at its MBC (Supplementary Figure 2.33).
Supplementary Figure 2.33. Percentage of PI-positive (membrane-disrupted) *E. coli* cells following treatment with various concentrations of SNAPP S16 at 0 ×, 0.5 × and 1 × MBC for 90 min. The bacterial cells were either used without prior treatment or pre-treated with a translation inhibitor, doxycycline, at 1 × its MIC (where MIC = 0.5 µg/mL). Error bars represent the standard deviation from the mean (n = 4).

Inhibition of protein synthesis does not affect SNAPP-induce membrane disruption.

In initial experiments, using flow cytometry to enumerate the number of viable bacterial cells, the bacterial cell counts for *E. coli* incubated with doxycycline (1 × its MIC) for 4 h remained the same as the starting inoculum. This was as expected as doxycycline is bacteriostatic against *E. coli*. Membrane disruption as measured by propidium iodide inclusion [3] was not affected when *E. coli* was incubated with doxycycline (Supplementary Figure 2.33). SNAPP S16 was found to induce membrane disruption at the same level in *E. coli* in the presence or absence of doxycycline, indicating that DNA translation and novel protein synthesis are not a requirement for SNAPP induced membrane disruption leading to cell death.
Supplementary Figure 2.34. Morphological studies of *E. coli* before and after treatment with S16 in MHB. a-g. Cryo-TEM images of *E. coli* before (a-c) and after incubation with S16 for 90 min at a lethal dose of 35 µg/mL (i.e., 1 × MBC of the unlabelled star in MHB) (d-g). Large aggregates (possibly aggregates of S16 with lysed cell contents) were observed around the rod-shaped *E. coli* cells (d-g). Hole formation (d), outer membrane fragmentation (e, f), and isolated cell fragments (g) were observed. Enlarged images of d-g are provided and the sections enlarged are boxed in red. Regions of interest are highlighted by red arrows. All images are representative of three independent experiments.
Cryo-TEM Images of S16-treated *E. coli* (with MEM as the Medium)

Supplementary Figure 2.35. Morphological studies of *E. coli* before and after treatment with S16 in MEM. a-k, Cryo-TEM images of *E. coli* before (a) and after incubation with S16 for 90 min at approximately 2 × MBC (*i.e.*, 15 µg/mL) (b-d) and 5 × MBC of the unlabelled star in MEM (*i.e.*, 35 µg/mL) (e-k). Damaged cells showed intracellular electron dense patches which were irregular in shape (b-k). Leakage of cellular contents and ruptured membranes (d-f, j) were observed. Enlarged images of a, b, and d are provided and the sections enlarged are boxed in red. All images are representative of three independent experiments.
Cryo-TEM Images of Antimicrobial Peptide-treated *E. coli* (with MHB as the Medium)

Supplementary Figure 2.36. Morphological studies of *E. coli* before and after treatment with melittin or ovispirin in MHB. a-c. Cryo-TEM images of *E. coli* before (a) and after incubation with melittin (b) or ovispirin (c) for 90 min at 1 × MBC. Damaged cells showed damaged cytoplasmic membranes with the outer membranes intact. Enlarged images are provided and the sections enlarged are boxed in red. All images are representative of three independent experiments.
### Supplementary Tables

#### Supplementary Table 2.1. Composition, Molecular Weight and Dispersity of the Star Peptide Polymers

<table>
<thead>
<tr>
<th>Peptide Polymer</th>
<th>Arm Number</th>
<th>Lysine:Valine, a:b&lt;sup&gt;a&lt;/sup&gt;</th>
<th>( M_n ) (kDa)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>( M_w ) (kDa)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>( D )&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before deprotection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S16&lt;sub&gt;Z&lt;/sub&gt;</td>
<td>16</td>
<td>1.83:1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S32&lt;sub&gt;Z&lt;/sub&gt;</td>
<td>32</td>
<td>1.97:1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>After deprotection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S16</td>
<td>16</td>
<td>1.83:1</td>
<td>43.8</td>
<td>127.9</td>
<td>2.9</td>
</tr>
<tr>
<td>S32</td>
<td>32</td>
<td>1.97:1</td>
<td>74.8</td>
<td>141.1</td>
<td>1.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by \(^1\)H NMR spectroscopic analysis.  
<sup>b</sup> The number-average (\( M_n \)) and weight-average (\( M_w \)) molecular weights and the dispersity (\( D \)) values were determined by aqueous GPC using a conventional calibration with PEG standards for the peptide polymers in the deprotected form.

**Note:**

The dispersity (\( D \)) values of SNAPPs as indicated by GPC analysis (Supplementary Table 2.1) are not necessarily a true reflection of the uniformity of SNAPPs due to potential interactions between the amphiphilic stars with the GPC columns causing tailing. Hence in this case, GPC analysis is not considered as the primary analytical tool but simply served as a routine inspection for SNAPPs. TEM and DLS analysis (Supplementary Figures 2.6-2.8) represent most accurately the uniformity of SNAPPs as they provide direct visualization of the nanoparticles in their native form and thus should be used primarily in judging the size distribution of the nanoparticles.
Supplementary Table 2.2. Killing Levels of SNAPPs

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Media</th>
<th>MBC (µM)(^a)</th>
<th>% reduction(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S16</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>MHB</td>
<td>0.72 ± 0.06</td>
<td>94.1</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.17 ± 0.01</td>
<td>98.7</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>MHB</td>
<td>1.42 ± 0.08</td>
<td>91.3</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.07 ± 0.04</td>
<td>99.8</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>MHB</td>
<td>1.54 ± 0.08</td>
<td>95.1</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.19 ± 0.05</td>
<td>96.7</td>
</tr>
<tr>
<td><em>A. baumannii</em></td>
<td>MHB</td>
<td>0.85 ± 0.05</td>
<td>92.9</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.05 ± 0.01</td>
<td>98.1</td>
</tr>
<tr>
<td><strong>MDR P. aeruginosa</strong></td>
<td>MHB</td>
<td>1.38 ± 0.03</td>
<td>96.8</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.08 ± 0.01</td>
<td>97.9</td>
</tr>
<tr>
<td><strong>MDR A. baumannii</strong></td>
<td>MHB</td>
<td>1.61 ± 0.23</td>
<td>95.7</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.05 ± 0.01</td>
<td>99.5</td>
</tr>
<tr>
<td><strong>S32</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>MHB</td>
<td>0.72 ± 0.54</td>
<td>94.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.05 ± 0.01</td>
<td>99.7</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>MHB</td>
<td>0.97 ± 0.05</td>
<td>95.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.02 ± 0.01</td>
<td>98.5</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>MHB</td>
<td>0.83 ± 0.14</td>
<td>99.2</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.08 ± 0.02</td>
<td>99.4</td>
</tr>
<tr>
<td><em>A. baumannii</em></td>
<td>MHB</td>
<td>0.79 ± 0.02</td>
<td>99.9</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.02 ± 0.01</td>
<td>99.2</td>
</tr>
<tr>
<td><strong>MDR P. aeruginosa</strong></td>
<td>MHB</td>
<td>1.00 ± 0.01</td>
<td>95.9</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.03 ± 0.01</td>
<td>96.9</td>
</tr>
<tr>
<td><strong>MDR A. baumannii</strong></td>
<td>MHB</td>
<td>0.85 ± 0.03</td>
<td>96.4</td>
</tr>
<tr>
<td></td>
<td>MEM</td>
<td>0.03 ± 0.01</td>
<td>98.3</td>
</tr>
</tbody>
</table>

\(^a\) The MBC listed in this column was determined over two independent experiments with two replicates for each variation in each experiment. \(^b\) The ‘% reduction’ was calculated by comparing the CFU/mL at \(x\) concentration to the CFU/mL of the untreated control at the end of a 90min incubation.
**Supplementary Table 2.3. MBCs of SNAPPs against Gram-Positive Pathogens**

<table>
<thead>
<tr>
<th>SNAPP</th>
<th>MBC(^a) (µM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(S.) <em>aureus</em></td>
<td>(S.) <em>mutans</em></td>
</tr>
<tr>
<td>S16</td>
<td>4.58 ± 1.13</td>
<td>3.55 ± 1.20</td>
</tr>
<tr>
<td>S32</td>
<td>2.23 ± 0.62</td>
<td>1.80 ± 0.14</td>
</tr>
</tbody>
</table>

\(^a\) All data are expressed as mean and standard deviations of four replicates \((n = 4)\) completed in two independent experiments.

**Supplementary Table 2.4. Antibiogram of MDR *P. aeruginosa* (FADDI-PA067)**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Susceptibility(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aztreonam</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Resistant</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>Resistant</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>Resistant</td>
</tr>
<tr>
<td>Colistin Sulfate</td>
<td>Resistant (&gt;110.8 µM)</td>
</tr>
</tbody>
</table>

\(^a\) Susceptibility of the bacteria species towards a particular antibiotic is interpreted based on resistant breakpoints provided by the Clinical and Laboratory Standards Institute [4].
### Supplementary Table 2.5. Antibiogram of MDR *A. baumannii* (FADDI-AB156)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µM)</th>
<th>Susceptibilitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>≥109.3</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>≥91.6</td>
<td>Resistant</td>
</tr>
<tr>
<td>Amoxicillin/Clavulanic Acid</td>
<td>≥87.6</td>
<td>Resistant</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>≥140.8</td>
<td>Resistant</td>
</tr>
<tr>
<td>Cefepime</td>
<td>≥133.2</td>
<td>Resistant</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>≥149.7</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>≥117.1</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>≥115.4</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≥12.1</td>
<td>Resistant</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>≥33.5</td>
<td>Resistant</td>
</tr>
<tr>
<td>Meropenem</td>
<td>≥41.7</td>
<td>Resistant</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td>≥137.8</td>
<td>Resistant</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>≥2149.8</td>
<td>Resistant</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>≥50.1</td>
<td>Resistant</td>
</tr>
<tr>
<td>Piperacillin/Tazobactam</td>
<td>≥247.3</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ticarcillin/Clavulanic Aid</td>
<td>≥333.0</td>
<td>Resistant</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>≥34.2</td>
<td>Resistant</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>≥55.1</td>
<td>Resistant</td>
</tr>
<tr>
<td>Trimethoprim/Sulfamethoxazole</td>
<td>≥1102.2</td>
<td>Resistant</td>
</tr>
<tr>
<td>Imipenem</td>
<td>≥213.8</td>
<td>Resistant</td>
</tr>
<tr>
<td>Colistin Sulfateb</td>
<td>13.8</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

*a* Susceptibility of the bacteria species towards a particular antibiotic is interpreted based on resistant breakpoints provided by the Clinical and Laboratory Standards Institute [4].
Supplementary Table 2.6. MICs of PAMAM G2 and G3 dendrimers

<table>
<thead>
<tr>
<th>PAMAM</th>
<th>MIC(^a) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>P. aeruginosa</td>
</tr>
<tr>
<td>G2</td>
<td>157.2</td>
</tr>
<tr>
<td>G3</td>
<td>&gt;148.2</td>
</tr>
</tbody>
</table>

\(^a\)MIC is defined as the minimum concentration of an antimicrobial agent at which no visible microbial growth is observed. Identical MIC values were obtained across four replicates completed in two independent experiments.

Supplementary Table 2.7. Composition, Molecular Weight, and Dispersity of the Linear Random Co-peptide Polymer L

<table>
<thead>
<tr>
<th>Peptide Polymer</th>
<th>Lysine:Valine, a:b(^a)</th>
<th>(M_n) (kDa)(^b)</th>
<th>(M_n) (kDa)(^c)</th>
<th>(M_w) (kDa)(^b)</th>
<th>(Đ)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>1.89:1</td>
<td>4.9</td>
<td>7.7</td>
<td>12.7</td>
<td>1.7</td>
</tr>
</tbody>
</table>

\(^a\)Determined from \(^1\)H NMR spectroscopic analysis of peptide polymer L. Ratio was the same as before deprotection. \(^b\)Determined from \(DP_n\) and the lysine:valine ratio obtained via \(^1\)H NMR spectroscopic analysis of peptide polymer L. \(^c\)Determined by aqueous GPC using PEG standards.

Note:

As mentioned above, the dispersity (\(Đ\)) values of the peptide polymer L as indicated by GPC analysis (Supplementary Table 2.7) are not necessarily a true reflection of the uniformity of L due to potential interactions between the amphiphilic peptide polymer L with the GPC columns causing tailing. Hence in this case, GPC analysis is not considered as the primary analytical tool but simply served as a routine inspection for L. TEM and DLS analysis (Supplementary Figure 2.13) represent most accurately the uniformity of L as they provide direct visualization of the nanoparticles in their native form and thus should be used primarily in judging the size distribution of the nanoparticles.
### Supplementary Table 2.8. MBCs of Linear Random Co-peptide Polymer L against a Range of Bacteria (in Nutrient-Rich Medium)

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MBC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>29.50</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>213.37</td>
</tr>
</tbody>
</table>

### Supplementary Table 2.9. Hemolytic Activity of SNAPPs

<table>
<thead>
<tr>
<th>Antimicrobial type</th>
<th>Code/Name</th>
<th>Media</th>
<th>HC₅₀ᵃ, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAPP</td>
<td>S16</td>
<td>MHB</td>
<td>58.3</td>
</tr>
<tr>
<td></td>
<td>S32</td>
<td>MHB</td>
<td>45.3</td>
</tr>
<tr>
<td>Linear analog</td>
<td>L</td>
<td>MHB</td>
<td>674.5</td>
</tr>
<tr>
<td>AMP</td>
<td>Ovispirin</td>
<td>MHB</td>
<td>61.8</td>
</tr>
<tr>
<td></td>
<td>Magainin II</td>
<td>MHB</td>
<td>81.1</td>
</tr>
<tr>
<td></td>
<td>Melittin</td>
<td>MHB</td>
<td>2.8</td>
</tr>
</tbody>
</table>

ᵃ HC₅₀ is the peptide concentration that results in 50% hemolysis.
Supplementary Table 2.10. Biocompatibility of SNAPPs (as Determined by YO-PRO-1/PI Apoptosis Assay)

<table>
<thead>
<tr>
<th>SNAPP</th>
<th>HEK293T</th>
<th>H4IIE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$</td>
<td>MBC$_{50}$</td>
</tr>
<tr>
<td>S16</td>
<td>2.78 / 0.027</td>
<td>102</td>
</tr>
<tr>
<td>S32</td>
<td>1.71 / 0.010</td>
<td>171</td>
</tr>
</tbody>
</table>

$^{a}$IC$_{50}$ is the SNAPP concentration that results in death in 50% of the cell population. $^{b}$MBC$_{50}$ refers to the SNAPP concentration that results in death in 50% of the bacterial cell population. The MBC$_{50}$ values here (rounded to the nearest two significant figures) were against CMDR A. baumannii. $^{c}$The % live cells at the highest concentration tested (i.e., 128 µg/mL or 1.71 µM) is 54.9 ± 0.3%. All experiments were conducted in minimal essential medium (MEM).

**Note:**

The adhered cells were incubated with SNAPPs for 90 minutes, stained with YO-PRO-1 and PI dyes, and analyzed by flow cytometry. Using YO-PRO-1 as an indicator of early apoptosis and PI as a measure of necrosis or cell death, viable cells were determined as cells that are negative for both YO-PRO-1 and PI.
Supplementary Table 2.11. MBCs of SNAPP S16 before and after fluorescent labelling against *E. coli*

<table>
<thead>
<tr>
<th>Peptide Polymer</th>
<th>MBC&lt;sup&gt;a&lt;/sup&gt; (µM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHB</td>
<td>MEM</td>
</tr>
<tr>
<td>S16</td>
<td>0.72 ± 0.06</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>AF488-S16</td>
<td>2.50 ± 0.03</td>
<td>0.39&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> All data are expressed as mean and standard deviations of four replicates (*n* = 4) completed in two independent experiments. <sup>b</sup> Identical results were obtained across all replicates.

**Note:**

The conjugation of AF488 to SNAPP S16 was found to slightly decrease its potency (3.5 and 2.2 times less effective against *E. coli* in MHB and MEM, respectively), likely due to the reduction of cationic amine groups. However, the interaction mechanism of the fluorescently tagged SNAPP with bacteria was expected to be similar to that before fluorescent tagging.

**References:**

Supplementary Figure 3.1. $^1$H NMR spectra (d$_6$-DMSO) of Cbz-protected star peptide polymers SB$_{16,Z}$ (a), SB$_{32,Z}$ (b), SR$_{16,Z}$ (c), SR$_{32,Z}$ (d), SH$_{16,Z}$ (e), and SH$_{32,Z}$ (f).
Supplementary Figure 3.2. $^1$H NMR spectra ($d_6$-DMSO) of deprotected star peptide polymers SB$_{16}$ (a), SB$_{32}$ (b), SR$_{16}$ (c), SR$_{32}$ (d), SH$_{16}$ (e), and SH$_{32}$ (f).
Supplementary Figure 3.3. DLS analysis of the star peptide polymers. a, DLS normalized mass % of the star peptide polymers as a function of hydrodynamic diameter ($D_H$). The numbers on the DLS distributions show the average $D_H$ of the polymers in water, each determined as an average of 30 measurements. b, The intensity autocorrelation curve corresponding to each DLS trace is shown.
Supplementary Figure 3.4. Synthesis of linear peptide polymers LH and LR. The ROP of lysine NCA only or mixtures of lysine NCA and valine NCA initiated by 4-methylbenzylamine, followed by deprotection of the carboxybenzyl groups on the lysine residues with HBr and subsequent dialysis in RO water to afford water-soluble linear peptide polymers LH and LR, respectively. The number of repeating units for lysine and valine are a and b, respectively. See also Table 3.1 for the lysine-to-valine ratios (i.e., a:b) of LR.
Supplementary Figure 3.5. a-d, $^1$H NMR spectra ($d_6$-DMSO) of linear Cbz-protected polymer LH$_Z$ (a), linear Cbz-protected polymer LR$_Z$ (b), deprotected polymer LH (c), and deprotected polymer LR (d). e, DLS normalized mass % of the linear peptide polymers as a function of hydrodynamic diameter ($D_{H}$). The numbers on the DLS distributions show the average $D_{H}$ of the polymers in water, each determined as an average of 30 measurements. f, The intensity autocorrelation curve corresponding to each DLS trace is shown.

Note on $^1$H NMR Spectra of the Linear Peptide Polymers:

For linear polymers LH and LR, the peaks associated with the 4-methylbenzylamine initiator could be observed, which enabled the number-averaged degree of polymerization ($D_{P_n}$) of the polymers to be determined. The $D_{P_n}$ values for LH and LR are 27 and 36 repeat units, respectively, both of which are close to the targeted value of 30.
Supplementary Figure 3.6. a-d, CD spectra of the star and linear peptide polymers (at a concentration of 0.2 mg/mL) in RO water with 0% v/v TFE (a), 20% v/v TFE (b), 50% v/v TFE (c), and 80% v/v TFE (d). The arrows point towards the troughs on the spectra for SR_{16} and SR_{32}, which are characteristic of $\alpha$-helices. Arrows with solid line: Characteristic troughs between 205 and 210 nm. Arrows with dotted line: Characteristic troughs at 220 nm. Inset: Expanded view of the CD spectra of LH and LR.
Supplementary Tables

Supplementary Table 3.1. Composition, Molecular Weights and Dispersity of the Star Peptide Polymers

<table>
<thead>
<tr>
<th>Star Peptide Polymer</th>
<th>Arm Number</th>
<th>Lysine:Valine, a:b&lt;sup&gt;a&lt;/sup&gt;</th>
<th>M&lt;sub&gt;n&lt;/sub&gt;(kDa)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>M&lt;sub&gt;w&lt;/sub&gt;(kDa)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Đ&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>SB&lt;sub&gt;16,Z&lt;/sub&gt;</td>
<td>16</td>
<td>2.45:1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SB&lt;sub&gt;32,Z&lt;/sub&gt;</td>
<td>32</td>
<td>2.87:1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SR&lt;sub&gt;16,Z&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16</td>
<td>1.83:1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SR&lt;sub&gt;32,Z&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32</td>
<td>1.97:1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SH&lt;sub&gt;16,Z&lt;/sub&gt;</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SH&lt;sub&gt;32,Z&lt;/sub&gt;</td>
<td>32</td>
<td>-</td>
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**Before deprotection**

<table>
<thead>
<tr>
<th>Star Peptide Polymer</th>
<th>Arm Number</th>
<th>Lysine:Valine, a:b&lt;sup&gt;a&lt;/sup&gt;</th>
<th>M&lt;sub&gt;n&lt;/sub&gt;(kDa)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>M&lt;sub&gt;w&lt;/sub&gt;(kDa)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Đ&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>SB&lt;sub&gt;16&lt;/sub&gt;</td>
<td>16</td>
<td>3.19:1</td>
<td>11.0</td>
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<tr>
<td>SB&lt;sub&gt;32&lt;/sub&gt;</td>
<td>32</td>
<td>2.87:1</td>
<td>15.2</td>
<td>44.8</td>
<td>2.9</td>
</tr>
<tr>
<td>SR&lt;sub&gt;16&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16</td>
<td>1.95:1</td>
<td>43.8</td>
<td>127.9</td>
<td>2.9</td>
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<tr>
<td>SR&lt;sub&gt;32&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32</td>
<td>2.00:1</td>
<td>74.8</td>
<td>141.1</td>
<td>1.9</td>
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<tr>
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<td>27.6</td>
<td>40.7</td>
<td>1.5</td>
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<td>SH&lt;sub&gt;32&lt;/sub&gt;</td>
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<td>-</td>
<td>56.3</td>
<td>88.7</td>
<td>1.6</td>
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</table>

<sup>a</sup> Determined by <sup>1</sup>H NMR spectroscopic analysis. <sup>b</sup> Determined by aqueous GPC using a conventional calibration with narrow molecular weight PEG standards for the deprotected star peptide polymers. <sup>c</sup> The synthesis and characterization of the polymers were reported in our earlier study [1].

Supplementary Table 3.2. Molecular Weights and Dispersity of the Linear Peptide Polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>M&lt;sub&gt;n&lt;/sub&gt;(kDa)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>M&lt;sub&gt;w&lt;/sub&gt;(kDa)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Đ&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>LH</td>
<td>6.2</td>
<td>8.8</td>
<td>1.4</td>
</tr>
<tr>
<td>LR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.7</td>
<td>12.7</td>
<td>1.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by aqueous GPC using PEG standards for polymers in the deprotected form. <sup>b</sup> The synthesis and characterization of the polymer were reported in our earlier study [1].
### Supplementary Table 3.3. MDCs, MBCs, and MICs of the Peptide Polymers against *S. mutans*

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Peptide Polymer</th>
<th>MDC (µM)</th>
<th>MBC (µM)</th>
<th>MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
<td><strong>SR&lt;sub&gt;16&lt;/sub&gt;</strong></td>
<td>0.4 ± 0.1</td>
<td>3.6 ± 1.2</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td><strong>SR&lt;sub&gt;32&lt;/sub&gt;</strong></td>
<td>0.3 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td><strong>LR</strong></td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

### Supplementary Table 3.4. HC<sub>50</sub> and HC<sub>50</sub>/MBC<sub>50</sub> of Reference Antimicrobial Peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>HC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (µg/mL)</th>
<th>HC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;/MBC&lt;sub&gt;50&lt;/sub&gt; (E. coli)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antimicrobial Peptide</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovispirin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27 (61.8)</td>
<td>15</td>
</tr>
<tr>
<td>Magainin II&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33 (81.1)</td>
<td>3</td>
</tr>
<tr>
<td>Melittin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0 (2.8)</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Multivalent Antimicrobial Peptide</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA-RWRW (25% graft)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34 (2.7)</td>
<td>17</td>
</tr>
<tr>
<td>PMA-RWRW (50% graft)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34 (1.8)</td>
<td>11</td>
</tr>
<tr>
<td>PMA-RWRW (100% graft)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34 (1.1)</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based upon extrapolation from Figure 3. <sup>b</sup> The amino acid sequences of ovispirin, magainin II and melittin are KNLRIIRKIIIIKKKYG-COOH, GIGKFLHSKKFGKAFVEIMNS-CONH₂, and GIGAVLKVLTTGLPALISWIKRKRQQ-COOH, respectively. The synthesis of the peptides and their antimicrobial activities and hemolytic activities are as previously reported [1]. <sup>c</sup> PMA-RWRW is constructed from the linking of the antimicrobial tetrapeptide RWRW to a polymaleic anhydride (PMA) scaffold at grafting ratios of 25%, 50%, and 100%, with molecular weights of 12600 Da, 18600 Da, and 30500 Da, respectively. The synthesis, characterization, antimicrobial activities, and haemolytic properties of these polymer-AMP conjugates are as reported by Kallenbach and co-workers [2].
Supplementary Table 3.5. Therapeutic indices of SR\textsubscript{16} and SR\textsubscript{32} (where MBC and MBC\textsubscript{50} were determined against \textit{E. coli} in minimal medium)

<table>
<thead>
<tr>
<th>Peptide Polymer</th>
<th>MBC\textsubscript{MEM}, (µM)</th>
<th>MBC\textsubscript{50,MEM} (µM)</th>
<th>Therapeutic index, (\frac{IC_{50}}{MBC_{50,MEM}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR\textsubscript{16}</td>
<td>0.17</td>
<td>0.09</td>
<td>31</td>
</tr>
<tr>
<td>SR\textsubscript{32}</td>
<td>0.05</td>
<td>0.03</td>
<td>57</td>
</tr>
</tbody>
</table>

References:

Supplementary Figure 5.1. Screening analysis for synergistic S16-antibiotic pairs. a-f. Log change in colony-forming units (CFU)/mL of E. coli (a), K. pneumoniae (b), P. aeruginosa (c), CMDR P. aeruginosa (d), A. baumannii (e) and CMDR A. baumannii (f) from time zero after treatment for 1.5 h with S16, a range of antibiotics, and combinations of S16 with the respective antibiotics, with all compounds at their MBC50. All data are expressed as mean ± standard deviation as indicated by the error bars (n = 3). **P < 0.01, ***P < 0.001, Student’s t test.
Supplementary Figure 5.2. Effects of SNAPPs on bacterial membranes. a, Uptake of propidium iodide (PI) by E. coli after treatment with SNAPP S16. E. coli cells were incubated with S16 for 90 min at 0.5 ×, 1 ×, and 2 × its MBC. After incubation the cells were stained with SYTO® 9 and PI nucleic acid dyes and subjected to flow cytometric analysis. Cells were determined to be ‘PI-positive’, and hence membrane-disrupted, if fluorescence emitted is captured by FL3 (indicated by the black box). Controls whereby no S16 was added (0 µM) were included. b, Membrane potential flow cytometric dot plots obtained after incubating E. coli with 30 µM DiOC$_2$(3) for 1 h in the presence/absence of CCCP (a proton ionophore), and SNAPP S16 at 0.5 ×, 1 ×, and 2 × its MBC. The controls where CCCP was either absent (-CCCP) or present (+CCCP) represent the normal membrane potential state and fully depolarized state for E. coli, respectively. A flow cytometry gate (black polygon) was drawn in each panel to indicate the position of the viable bacteria in the absence of CCCP or SNAPP.
**Supplementary Figure 5.3.** a-e, Log change in CFU/mL of various bacterial species from time zero after treatment for 1.5 h with S16 at its MBC50 and a range of antibiotics at the indicated concentrations. The synergistic pairs investigated are S16-ampicillin (Amp) against *P. aeruginosa* (a), S16-AgNO$_3$ (Ag$^+$) against CMDR *P. aeruginosa* (b), S16-doxycycline (Dox) against CMDR *P. aeruginosa* (c), S16-AgNO$_3$ (Ag$^+$) against CMDR *A. baumannii* (d), and S16-imipenem (Imi) against CMDR *A. baumannii* (e). All data are expressed as mean ± standard deviation as indicated by the error bars ($n = 3$). ***$P < 0.001$, **$P < 0.05$, Student’s $t$ test.

**Legend:**
- Untreated
- Treated groups:
  - S16 at MBC50
  - Antibiotic
  - Antibiotic + S16 at MBC50
Supplementary Figure 5.4. a-e. Log change in CFU/mL of various bacterial species from time zero after treatment for 1.5 h with S16 at its MBC25 and a range of antibiotics at the indicated concentrations. The synergistic pairs investigated are S16-ampicillin (Amp) against P. aeruginosa (a), S16-AgNO₃ (Ag⁺) against CMDR P. aeruginosa (b), S16-doxycycline (Dox) against CMDR P. aeruginosa (c), S16-AgNO₃ (Ag⁺) against CMDR A. baumannii (d), and S16-imipenem (Imi) against CMDR A. baumannii (e). All data are expressed as mean ± standard deviation as indicated by the error bars (n = 3). ***P < 0.001, **P < 0.05, Student’s t test.
Supplementary Figure 5.5. a-e. Log change in CFU/mL of various bacterial species from time zero after treatment for 3 h with S16 at its MBC25 and a range of antibiotics at the indicated concentrations. The synergistic pairs investigated are S16-ampicillin (Amp) against P. aeruginosa (a), S16-AgNO₃ (Ag⁺) against CMDR P. aeruginosa (b), S16-doxycycline (Dox) against CMDR P. aeruginosa (c), S16-AgNO₃ (Ag⁺) against CMDR A. baumannii (d), and S16-imipenem (Imi) against CMDR A. baumannii (e). All data are expressed as mean ± standard deviation as indicated by the error bars (n = 3). ***P < 0.001, **P < 0.05, Student’s t test.
Supplementary Tables

Supplementary Table 5.1. Minimum Bactericidal Concentrations (MBCs) of S16 and Antibiotics

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>E. coli</th>
<th>K. pneumoniae</th>
<th>P. aeruginosa</th>
<th>CMDR P. aeruginosa</th>
<th>A. baumannii</th>
<th>CMDR A. baumannii</th>
</tr>
</thead>
<tbody>
<tr>
<td>S16</td>
<td>0.7</td>
<td>1.5</td>
<td>1.4</td>
<td>1.4</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>3.5</td>
<td>10.6</td>
<td>1.2</td>
<td>38.3</td>
<td>8.2</td>
<td>10.6</td>
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<tr>
<td>Amp</td>
<td>9.4</td>
<td>655.1</td>
<td>&gt;5860.0</td>
<td>&lt;1.4</td>
<td>&gt;2930.7</td>
<td>&gt;2930.7</td>
</tr>
<tr>
<td>Gen</td>
<td>3.7</td>
<td>2.2</td>
<td>2.4</td>
<td>&lt;1.1</td>
<td>12.1</td>
<td>&gt;1104.5</td>
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<tr>
<td>Tob</td>
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<td>NT⁺</td>
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<tr>
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<td>50.7</td>
<td>629.7</td>
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<td>NT⁺</td>
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_nt = Not tested._