Bringing together engineering and entrepreneurship: Antimicrobial effectiveness of tethered C-CHY1 on antibiotic resistant bacteria

A Dissertation

Submitted to the Faculty of

Worcester Polytechnic Institute

In Partial Fulfillment of the Requirements for the

Degree of

DOCTOR OF PHILOSOPHY in

Innovation and Entrepreneurship in Biomolecular Engineering

July 2019

By

Todd E. Alexander

Terri A. Camesano, Ph.D.
Professor, Research Advisor
Department of Chemical Engineering
Worcester Polytechnic Institute

Frank Hoy, Ph.D.
Professor and Director
Foisie Business School
Worcester Polytechnic Institute

Tanja Dominko, Ph.D., D.V.M.
Associate Professor
Department of Biology &
Biotechnology
Worcester Polytechnic Institute

Jerrold Shapiro, Ph.D.
President and CEO Fem Medical LLC

Kathleen Swana, Ph.D.
Physical Scientist
Combat Capabilities Development
Command Soldier Center

Bringing together engineering and entrepreneurship: understanding the role of tethered C-CHY1 in the fight against antimicrobial resistance

Table of Contents

Contents

Table of Contents i
Abstract vi
Acknowledgements x
Authorship xiii
Abbreviations xvi
Chaper 1: Specific Aims 1
1.1 Specific Aims 2
References 5
Chapter 2 Background 11
2.1 Background 12
References 23
Chapter 3 QCM-D Characterization of Time-Dependence of Bacterial Adhesion 36
Abstract 37
3.1 Introduction 38
3.1.1 Techniques to Measure and Characterize Bacterial Adhesion 39
3.1.2 Strategies to Prevent Bacterial Adhesion and Biofilm Formation 40
3.2 Factors affecting bacterial adhesion and biofilm maturation evaluated using QCM-D 42
3.2.1 QCM-D Technique and Sensitivity 42
3.2.2 Studies of bacterial adhesion factors using QCM-D 43
3.3. QCM-D Evaluation of Modified Surfaces that Affect Bacterial Adhesion 46
3.3.1 Passive strategies to prevent bacterial adhesion 47
3.3.2 Active strategies to prevent bacterial adhesion 48
3.4 Methods and Modelling of QCM-D Systems: Current Status and Future Directions 49
3. 4.1 Models based on change in frequency 49
3.4.2 Models based on change in frequency and energetic losses (dissipation) 51
3.4.3 Models based on ΔD vs. Δf ΔD / Δf 53
3.5 Conclusions/Outlooks 55
References 57

Specific Aim 1: Chapter 4 Mechanistic Analysis of Tethered Antimicrobial Peptide Chrysophsin-1 as a Function of Tether Length Using QCM-D 66
Abstract 67
4.1 Introduction 68
4.2 Experimental and/or Theoretical Methods 70
4.2.1 Bacterial Strains and Culturing. 70
4.2.2 Peptides. 71
4.2.3 QCM-D: Covalent Linking of C-CHY1. 71
4.2.4 QCM-D Control Experiments: Physical Adsorption of CHY1. 72
4.2.5 Bacterial Mortality. 73
4.2.6 QCM-D: Data Modeling. 73
4.3 Results 75
4.3.1 Addition of Mass Suggests Successful Grafting of C-CHY1 to the Surface via PEG Tethers. 75
4.3.2 Film Thickness Increases with Tether Length. 77
4.3.3 PEG Tether Mass Plus Associated Water Molecules Determine the Areal Mass of the Film. 79
4.3.4 The Longest Tether Length Demonstrates the Highest Bacterial Mortality and is Independent of Bacteria Type. 80
4.3.5 Control Experiments Demonstrate a Baseline of Approximately 25% Mortality. 81
4.4 Discussion 82
4.4.1 Proposed Mechanism for Tethered C-CHY1 Activity 82
4.4.2 Proposed mechanism for Physically-adsorbed peptide, CHY1 83
4.4.3 Proposed mechanism for C-CHY1 tethered with PEG 866 84
4.4.4 Proposed mechanism for C-CHY1 tethered with PEG 2000 85
4.4.5 Proposed mechanism for C-CHY1 tethered with PEG 7500 86
4.5 QCM-D as a Tool for the Study of Tethered AMPs 86
4.6 Conclusions 89
REFERENCES 90
4.7 Supporting Information 96
Supplemental References 102
Specific aim 2: Chapter 5 Role of lipopolysaccharide and lipoteichoic acid on C-CHY1 interactions with model Gram-Positive and Gram-Negative Bacterial Membranes 103
Abstract 104
5.1 Introduction 105
5.2.1 Lipids, Lipid Ratios and Peptide Solutions 108

5.2.2 Other Reagents/Buffers 110
5.2.4 QCM-D Supported Lipid Bilayer Formation 110
5.2.5 Data analysis 111
5.3 Results and Discussion 112
***5.3.2** Increased temperature does not increase interaction dynamics with Gram-positive membranes but alters the overall mechanism of C-CHY1* 114
***5.3.5.** Overall changes in bilayer thickness reveal mechanistic differences between Gram-positive and Gram-negative bacterial model membranes.* 119
***5.3.7.** Proposed mechanism of C-CHY1 against bacterial membranes and implications for C-CHY1 clinical applications.* 123
References 128
Supplementary Data 137
PEG deposition through QCM-D 137
Antimicrobial Activity 142
Representative Frequency and Dissipation Trends for Gram-positive and Gram-negative Supported Lipid Bilayers 144
Specific Aim 3: Chapter 6 Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program 146
6.1 Introduction 148
6.2 Growth and Current state of entrepreneurship Education 150
6.3 Barriers to Implementation of Entrepreneurship in STEM Education 151
6.3.1 Faculty and Institutional Barriers 152
6.3.2 Student Barriers 154
6.4 Methods 154
6.5 Results 155
6.5.1 Why Develop an Interdisciplinary Education Program for STEM Students? 156
6.5.2 For Whom Will the Program Be Designed and Offered? 157
6.5.3 For Which Results is the Interdisciplinary Education Program Evaluated? 157
6.5.4 What Content and Theories Will Be Addressed? 160
6.5.5 How Will the Program Be Operated in Order to Achieve its Objectives? 161
6.6 Lessons Learned 161
6.6.1 What faculty strategies are effective for introducing entrepreneurship concepts to STEM students? 162
6.6.2 How might student acceptance of entrepreneurship be gained? 163

6.6.3 What positive institutional impacts might be derived from introducing entrepreneurship into STEM education programs? 166
6.7 Conclusions 169
References 170
Specific Aim 3: Chapter 7 Success factors and challenges of grassroots innovations: Learning from failure 178
Abstract 179
7.1 Introduction 179
7.2 Background 181
7.1.1Grassroots and inclusive innovations 181
7.2.2 The role of protective niches 184
7.2.3 Main challenges of grassroots innovation projects 185
7.2.4 Useful theoretical paradigms for a strategic diagnostic of grassroots projects 187
7.2.5 Innovation adoption/diffusion 188
7.2.6 Market forces 189
7.3 Methodology 192
7.3.1 Data coding 194
7.4 Case study: The Paper Block Project in the Informal Katutura Settlement 195
7.4.1 The problems 195
7.4.2 The initial project 196
7.4.3 Redesigning the product and improving the project 198
7.4.4 Recommendations for scaling up the project 200
7.5 Findings 202
7. 6 Discussion 204
7.7 Implications 207
7.7.1 Theoretical implications 207
7.7.2 Practical implications 208
7.8 Conclusion 208
References 212
Specific Aim 3: Chapter 8 Utilizing the Lean Startup Methodology and Business Model Canvas as Tools to Chart a Path to Market for AMP Based Technologies 218
8.1 A path to market for AMP based technologies 219
8.2 References 229
Business Plan 235

Chapter 9 Conclusions and Future Directions 264
9.1 Conclusions 265
9.2 Future Directions 269
References 273
Supplemental Figures 288

Abstract

Healthcare associated infections (HAIs) cost the US healthcare system over $45 billion to treat and cause millions of deaths annually. A large subset of HAIs are associated with medical devices that are meant to improve and save lives. Infected devices are treated using traditional antibiotics, contributing to development of antibiotic resistance. Antibiotic resistance is expected to cost $100 trillion and kill more people a year than cancer by 2050; thus, new alternative antimicrobials for the treatment of device-associated HAIs are critically needed.

Antimicrobial peptides (AMPs), such as 26 amino-acid (aa), marine-derived Chrysophsin-1 (CHY1), are poised to reduce HAIs due to their broad antimicrobial activity and unique mechanisms of action that do not promote bacterial resistance. AMPs are short (12-50aa), positively charged (+2-+9) proteins found in the innate immune systems of many different species. Their high separation of hydrophilic and hydrophobic residues leads to many unique mechanisms derived from many unique secondary and tertiary structures that are not yet well understood.

Despite the discovery of over 2000 natural AMPs and many more synthetically designed AMPs, none have been successfully commercialized for healthcare applications due to challenges surrounding cytotoxicity, short *in vivo* half-life (degradation), high costs of production and effectiveness in physiological environments (such as those with high-salt). Several strategies have been investigated to overcome these challenges, for example, truncation of cytotoxic sequences or D-amino acid substitution to improve AMP toxicity and stability; however, many of these strategies can reduce antimicrobial effectiveness. A unique strategy of increasing stability, reducing cytotoxicity, and maintaining antimicrobial activity that is relevant for medical devices is the covalent *tethering* (binding) of AMPs via a flexible tethering molecule to the surface. However, the effect of tethering parameters on resulting AMP mechanisms and activity is still widely debated. AMP activity can vary widely by utilizing different tethering strategies, which include additional variables such as: (1) peptide choice and properties (such as native mechanism, concentration, charge, and structure), (2) tether

choice and properties (such as chemical composition, length, charge, surface density, and flexibility), and (3) testing conditions (such as temperature, solvent composition and substrate type).

Some studies suggest that AMP performance may be tether-dependent, for example some AMPs require longer tethers while others do not and some need a flexible tether. Thus, models for predicting successful tethering strategies for different AMP properties, which currently do not exist, must be developed. Further, complicated and often destructive techniques, such as XPS and SEM, are typically implemented to study the relationship of all these parameters vs. antimicrobial activity, which are labor-intensive and limited in scope. Predictive models guiding tether strategy need to be constructed, but also new techniques to study tethering be developed. If these technical milestones are achieved they can serve as a predicate for commercial implementation of a host of new therapies targeted at reducing device-associated HAIs.

The overall goal of this thesis was to study the relationship between antimicrobial activity of tethered C-CHY1 examining both spacer length and peptide surface density and the development of a feasible clinical business case for tethered AMPs. To achieve this goal, a traditional entrepreneurial approach was taken in which a minimally-viable product was first designed and business case analyzed, followed by studies to better optimize and understand the underlying structure-mechanism relationships. CHY1 with a C-terminus cysteine to allow for surface-binding (C-CHY1) was tethered onto a silicon dioxide surface via a flexible poly(ethylene glycol) (PEG) tether, and then both surface binding behavior and antimicrobial success of C-CHY1 were examined as a function of tether properties and reaction conditions. For these studies, quartz-crystal microbalance with dissipation (QCM-D) was the primary technique, a real-time, non-destructive flow method that was then coupled with downstream characterization techniques: fluorescent microscopy and contact angle measurements. In parallel a deep dive into domestic and international business models for commercializing AMP technologies.

Specifically, tether length and surface density effects on C-CHY1 mechanisms were studied, followed by the effect of temperature, type of microbe, and salt concentration on the antimicrobial

mechanisms of tethered C-CHY1. QCM-D was used to measure binding of C-CHY1 via three different length tethers, PEG molecular weight (MW) 866, 2000 and 7500, followed by microscopy to measure antimicrobial effectiveness against two model microbes *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). Modeling of QCM-D data allowed for surface density and thickness to be calculated and related to C-CHY1 antimicrobial activity. PEG 7500 allowed proper C-CHY1 orientation and mobility, allowing for its native pore-forming mechanism and highest activity while PEG 866 tethers led to denser grafting and an effective, yet non-native ion displacement mechanism. The QCM-D was used to characterize the effect of salt concentration and temperature reaction conditions on the grafting density of C-CHY1 tethered via PEG 866 and PEG 7500, which was then related to antimicrobial activity. For PEG MW 866, neither temperature nor salt concentration increases significantly altered the grafting density of C-CHY1 while for PEG 7500 increasing temperature allowed for significantly increased grafting density. C-CHY1 density had no significant effect on antimicrobial activity against either microbe. Temperature of bacterial incubation did demonstrate microbe-specific changes in C-CHY1 antimicrobial activity. These results demonstrated that small changes in reaction conditions can drastically change membrane selectivity of C-CHY1.

An in-depth investigation of the effects of bacterial membrane composition and temperature on soluble C-CHY1 mechanism was implemented to better understand the molecular membrane- and temperature-dependent selectivity and structure-function of C-CHY1. Supported lipid bilayers (SLBs) formed in QCM-D can be used as model membranes to elucidate AMP action mechanisms against membranes of different compositions. Two and three component SLBs representative of Gram-negative phosphatidylethanolamine (PE) and phosphatidyglycerol acid (PG) with and without charged lipopolysaccharide, LPS and Gram-positive bacteria phosphatidylcholine (PC) and PG with and without charged lipoteichoic acid, (LTA) were formed at both 23°C and 37°C. C-CHY1 at 5 μM was exposed to the different membranes and mechanistic surface action was studied. The membranes formed highly different baseline responses in QCM-D, indicative of vastly different membrane structures, thicknesses and deposition

behaviors on SiO_2, warranting future studies. Further, significant effects of LTA incorporation were observed in both peptide interaction and deposition. There were measurable effects of temperature on membrane formation as well as peptide interaction kinetics and even mode of interaction.

Lastly, business models for the commercialization of novel medical device technologies such as surface-tethered C-CHY1 were investigated. While this technology has the potential to solve many unmet needs, there must a commercialization plan implemented in order to have an impact. There is a clear disconnect between technology development in academia and technology commercialization in industry that must be connected. Development of an entrepreneurial mindset at the graduate school level, can help bridge the gap. A thorough investigation of domestic and international business models for commercializing AMP technologies was carried out and distilled in the form of the Business Model Canvas developed by Alexander Osterwalder that can be used as a roadmap for commercialization efforts. **Using the QCM-D a relationship between both spacer length and peptide surface density and the antimicrobial activity of tethered C-CHY1 was determined. A business plan was developed in order to increase the impact of this and other AMP based work. This work provides a roadmap for future researchers to quickly develop and commercial novel AMP based coating technology.**

Acknowledgements

"It's like in the great stories, Mr. Frodo. The ones that really mattered. Full of darkness and danger they were. And sometimes you didn't want to know the end…because how could the end be happy? How could the world go back to the way it was when so much bad had happened? But in the end, it's only a passing thing…this shadow. Even darkness must pass. A new day will come. And when the sun shines it will shine out the clearer. Those were the stories that stayed with you. That meant something.
Even if you were too small to understand why. But I think, Mr. Frodo, I do understand. I know now. Folk in those stories had lots of chances of turning back only they didn't. Because they were holding on to something. Frodo: What are we holding on to, Sam? Sam: That there's some good in this world, Mr. Frodo. And it's worth fighting for."
-J.R.R. Tolkien, The Two Towers

While there were some days that felt that I was never going to finish there were so many people around me, like Sam, that helped me get to the end. Without the support of so many people I wouldn't be writing this part of my thesis or any of it at all.

I want to thank Dr. Terri Camesano for being a patient and supportive advisor. You were always there when I needed you and you knew when to push me. I know how important family is to you and I know you know how important it was to me. I want to thank you for allowing me to take the time to go see my siblings various sporting events and activities. It meant the world to me!

To my co-advisor Dr. Frank Hoy, I want to thank you for igniting my passion for innovation and entrepreneurship. I look back to the first IGERT course and see how far I have come from then to now. I have learned a lot under your mentorship. I know I still have a lot to learn in this area and I thank you again for your guidance. I hope to carry on the flame you ignited and spread the knowledge of entrepreneurial training in graduate studies. As you know some of my siblings have gone to WPI with degrees in the business field. They cannot say enough good things about you as well.

I am so thankful to have signed up for the 5 Minute Pitch Contest in 2014 because that is where I was introduced to Dr. Jerry Shapiro. I still remember our first meeting where you blew me away with everything I needed to do to make a great pitch. While we didn't win that year our pitch was definitely well received. I want to thank you for being on my committee and to thank you for being a fantastic mentor for both Lindsay and I with AMProtection. Again I cannot thank you and Frank enough for sparking my entrepreneurial interests.

To Dr. Tanja Dominko I want to thank you for being a great advisor as well. I may not have had as many formal committee meetings as I should have I really valued our conversations every time we bumped into one another. You made me think of questions that were outside of my comfort zone and my project was better for that.

Dr. Kathleen Swana, or just Kathleen when I first met you, thank you for setting an amazing example of what a graduate student should be. I looked up to you from day one of starting in the lab. I want to thank you for the help with the interpretation of the polar plots and all the other great advice you have given me over the years.

While on the subject of polar plots I would also like to thank Dr. Ramanathan Nagarajan for help with interpreting them as well. I would also like to thank him for taking the time to meet with me on multiple

occasions and honestly I can pinpoint a meeting with you as the start of the end in helping me get the ball rolling on writing my thesis.

I have been at WPI for so long that if I listed everyone by name that I needed to thank I think this portion of the document would be longer than the rest so I will try to be brief. I would like to thank the entire Chemical Engineering Department, professors, administrators, support staff and students for making my graduate experience a good one. I would like to thank Felicia, Tiffany, and Leslie for handling all the issues I brought them. Thank you Doug and Tom for repairing some of our equipment that has seen better days.

Thank you to Andy Butler, Eric Sabacinski, Elizabeth Stepien, Daryl Johnson, Tina Stratis, Cynthia Gillespie and everyone at Gateway Park that helps the building run in one way or another. Thank you to Jimmy and Bill as well! I really enjoyed our conversations.

I would like to thank the Gericke Lab for the use of the Malvern Zetasizer Nano ZS.

I would also like to thank Glenn Gaudette, Janet Zafiris, and Curtis Abel for allowing me to work with them on implementing the KEEN grant. It was a pleasure working with you and that and is a program that I am glad I could help support in any capacity.

Thank you to Debbie Baron, Joan Murphy, Camille Bouchard-Chhoeuk, Rory Flinn and anyone else I may have missed in the Office of the Provost. Thank you Todd Keiller, Sarah Mahan, and Gina Betti for all your support on everything related to innovation and entrepreneurship.

I would like to thank all the advisors of AMProtection as well as some of your advice influenced my thesis. I would also like to thank the Venture Forum and their members for their support. In particular I would like to thank Joe Vignally who ran the forum for most of the years I was active.

For the sake of brevity I cannot list everyone, but I will thank you a second time, thank you to all my colleagues in the Chemical Engineering Department you made my experience better! Thank you to everyone in Gateway both professors and students. The wonderful conversations I had with you all helped me get through the day. I am seriously impressed by all of the amazing work you do and I consider myself lucky to work in the same building as all of you incredible people.

I would like to thank all the members of the Camesano Lab past and present. To Ziqi I know I am leaving the lab in good hands! Thank you to Lindsay, Ellie, Prachi, Sarah, Roberta, Sawinee, Kathleen, Paola, Christina, Mary, Alicia, Ivan, and Alec. To all the students in the MQPs I mentored I want to say that you all impressed me with your drive, your passion and your scientific curiosity. WPI students really are the best students. I would like in particular to thank Zach Lipsky and Ian Smith for their help in the lab and without their help this thesis would not be what it is. I also am happy to say that I did not scare you away from pursing Ph.D.s yourselves! Thank you to Julien and Kellie for being great friends to me when I first got here, the first few years flew by. If I forgot anyone I swear I didn't mean to but sometimes the years have blended together for me.

While technically not in the Camesano Lab (Rolle Lab, close enough) I do have to thank Dalia whose positivity helped me get through the tough times at the end.

There is no way I could have finished my Ph.D. without the support of my friend and former colleague Lindsay. Your work ethic was truly incredible and an inspiration for me, I can seriously hope one day to be

even half as productive as you in the lab. You were always there for me when I needed it including being crazy enough to start a business with me. Even though that didn't work out the way I know we wanted it to, I am just happy to have a great friend and the end of the day.

I would like to thank my primary funding sources Worcester Polytechnic Institute, the National Science Foundation IGERT program (NSF DGE 1144804), and the WPI Kern Entrepreneurial Engineering Network (KEEN) grant.

Lifting was like therapy for me and to no surprise the WPI gym is one of my favorite places on campus…what can I say the Ph.D. is stressful. I would like to thank all of my lifting friends for keeping me both sane and strong. Thank you Chuan Tian, Tom Blais, Dave Deisadze, Blaine Bursey… and I am going to cut the list off here or it may go on for a page two but to those not listed thank you. I would also like to thank all my Cross Country and Track friends (pretty much all my undergrad friends as well) for supporting me and still running with me even though I am super slow now.

To my family thank you for always being there for me and being so supportive. I have definitely tried to be the best oldest sibling to you that I can be although I know I am not perfect. When times got really rough and I wanted to quit I thought of what kind of example that would set. I knew I could not let you down and that I had to keep moving forward. When I first came to WPI 12 years ago as a freshman part of the reason I picked WPI was so that I could still be close to you and come home for sports, birthdays and other family events. I can probably say that was one of the best decisions I have made in my life. Watching you all grow up has been incredible and I am so proud of you all! I just hope that one day you will be as proud of me as I am of all of you! I could keep going here but the long and the short of it is without you this thesis would not have happened and its okay you can keep calling me a nerd there is nothing wrong with that.

Dad thank you for also being supportive throughout the years and instilling the importance of education and hard work!

Mom you are my hero! You are the strongest person I know and I am who I am today because of you! You really did teach me the true meaning of what it means to be strong and keep going in tough times. There are too many things I want to say here but every time I think about what I want to say I cry. I guess all I can say is just like that first marathon you have been by my side from the first step, just like in the marathon you have been there for me every step of this thesis even if metaphorically. The love and support you have given me throughout my life has been incredible! I know I have said this a few times already but there is no way this document is submitted without your support!

Last but not least, thank you to my incredible wife Kelsey! One could argue the most important thing that ever happened at WPI for me was meeting you! You endured my late nights, weird hours, obsessive gym habits, and well me. I have no idea what I would do without you, you make my life so much better! While I could write a dissertation about how incredible you have been throughout the Ph.D., simply put I could not have done it without you. Your family is also just as incredible as you and their support also meant the world to me. Some of the best nights over the years are the nights where we make each other laugh over the silliest little things and cannot stop. Those nights really made getting through the Ph.D. much easier.

This thesis is dedicated to my mom, my hero.

Authorship

All of the work here is my own except for the Collaborations noted.

Chapter 3: Todd E. Alexander[a], Lindsay D. Lozeau[a], Terri A. Camesano[a]

Chapter 4: Lindsay D. Lozeau[a*], Todd E. Alexander[a*], Terri A. Camesano[a]

Chapter 5: Todd E. Alexander[a], Lindsay D. Lozeau[a], Ian M. Smith[a], Zachary Lipsky[b], Terri A. Camesano[a]

Chapter 6: Todd E. Alexander[a], Lindsay D. Lozeau[a], Terri A. Camesano[a], Frank Hoy[c]

Chapter 7: Léo-Paul Dana[d], Calin Gurău[d], Frank Hoy[c], Veland Ramadani[e], Todd Alexander[a]

Chapter 8: Todd E. Alexander, Frank Hoy[c]; Business Model Canvases Todd E. Alexander[a], Lindsay D. Lozeau[a] and Business Plan Lindsay D. Lozeau[a], Todd E. Alexander[a]

[a] *Chemical Engineering, Worcester Polytechnic Institute, Worcester, MA*

[b] *Biomedical Engineering, Worcester Polytechnic Institute, Worcester, MA*

[c] *Foisie Business School, Worcester Polytechnic Institute, Worcester, MA*

[d] *South-East European University, Macedonia*

[e] *Montpellier Business School, France*

**Co-primary first authors on the publication*

Publications

Lozeau, L.D., **T.E. Alexander**, and T.A. Camesano, *Proposed mechanisms of tethered antimicrobial peptide chrysophsin-1 as a function of tether length using QCM-D.* The Journal of Physical Chemistry B, 2015. **119**(41): p. 13142-13151.

Alexander, T.E., L.D. Lozeau, and T.A. Camesano, *QCM-D Characterization of Time-Dependence of Bacterial Adhesion.* The Cell Surface, 2019: p. 100024.

Alexander, T.E., Lozeau, Linsday D., Camesano, Terri A., Hoy, Frank, *Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program.* Portland International Center for Management of Engineering and Technology (PICMET), 2019.

Dana, L. P., Gurău, C., Hoy, F., Ramadani, V., & Alexander, T. (2019). Success factors and challenges of grassroots innovations: Learning from failure. *Technological Forecasting and Social Change*.

Conferences

Alexander, T.E., Lozeau, L.D., Camesano, T.A. (2014) Effect of spacer length on the antimicrobial activity of the bound peptide, chrysophsin-1. *Society for Biomaterials Conference Proceedings*, Denver, CO.

Alexander, T.E.; Lozeau, L.D.; Camesano, T.A. (AUG 2015) Mechanistic study using a quartz crystal microbalance: Effect of spacer length on the antimicrobial activity of the bound peptide, Chrysophsin-1. American Chemical Society National Meeting, Boston MA.

Alexander, T.E.; Lozeau, L.D.; Camesano, T.A. (FEB 2016) Effect of Density and Spacer Length on the Mechanism of Action of the Tethered Peptide Chrysophsin-1. The Graduate Research Innovation Exchange, Worcester MA.

Alexander, T.E.; Lozeau, L.D.; Camesano, T.A. (MAY 2016) The immobilization and activity of Chrysophsin-1 antimicrobial peptide onto catheter surfaces, World Biomaterials Congress, Montreal, Quebec, Canada.

Alexander, T.E.; Lozeau, L.D.; Camesano, T.A. (AUG 2015) Mechanistic study using a quartz crystal microbalance: Effect of spacer length on the antimicrobial activity of the bound peptide, Chrysophsin-1. American Chemical Society National Meeting, Boston MA.

Alexander, T.E.; Lozeau, L.D.; Lipsky, Z.; Camesano, T.A. (SEPT 2016) Effect of tether length and density on tethered antimicrobial peptide, Chrysophsin-1 antimicrobial mechanisms, The 6th Thesinge biofilm meeting, Thesinge, The Netherlands.

Alexander, T.E.; Lozeau, L.D.; Lipsky, Z.; Camesano, T.A. (SEPT 2016) Effect of Tether Length and Density on Tethered Antimicrobial Peptide, Chrysophsin-1 Antimicrobial Mechanisms of Q-Sense QCMD (Quartz Crystal Microbalance with Dissipation) User Meeting in Cambridge, MA.

Alexander, T.E.; Lozeau, L.D.; Zachary Lipsky; Camesano, T.A. (FEB 2017) Effect of Density and Spacer Length on the Mechanism of Action of the Tethered Peptide Chrysophsin-1. The Graduate Research Innovation Exchange, Worcester MA.

Abbreviations

ΔD	**Change in dissipation**
δ_f	Decay lengths of the acoustic wave in the bulk film
δ_L	Decay lengths of the acoustic wave in the bulk liquid
Δf	Change in frequency
Δm	Change in mass
η_L	Viscosity of the bulk liquid assumed to be water (kg/m·s)
ρ_q	Density of quartz, 2.648 g/cm^3
ρ_f	Film density (kg/m^3)
µM	micro-molar
μ_q	Shear modulus of the crystal, 2.947x10^{11} g/cm·s^2
A	Active crystal surface area
AFM	Atomic Force Microscopy
APTMS	(3-Aminopropyl)trimethoxysilane
AMP	Antimicrobial peptide
AT cut	Temperature compensated cut, temperature has minimum effect on QCM performance
BMC	Business Model Canvas (Alexander Osterwalder)

C	Sauerbrey Constant
CAUTI	Catheter associated urinary tract infection
CDC	Centers for Disease Control and Prevention
CHY1	Chrysophsin-1 FFGWLIKGAIHAGKAIHGLIHRRRH
C-CHY1	Cysteine modified Chrysophsin-1 CFFGWLIKGAIHAGKAIHGLIHRRRH
Cpx	Signaling pathway
CVC	Central Venous Catheter
ECM	Extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substance
ESEM	Environmental scanning electron microscopy
FDA	U.S. Food and Drug Administration
f_0	Fundamental frequency of the quartz crystal
G'	Storage modulus of the film attached to the crystal surface
G''	Loss modulus
GAIN	Generating Antibiotic Incentives Now Act
HAI	Healthcare-associated infections
I-Corps	National Science Foundation Innovation Corps Program

IGERT	Integrative Graduate Education and Research Traineeship*: Training Innovative Leaders in Biofabrication*
IQP	Interactive Qualifying Projects
LPS	Lipopolysaccharides from Escherichia coli O55:B5 purified by phenol extraction
LOS	Patient length of stay
LTA	Lipoteichoic acid from Staphylococcus aureus bacterial cell wall polymer
$\mathbf{m_q}$	Mass liquid
$\mathbf{m_f}$	Mass film
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSR	Men on the Side of the Road
NPV	Net Present Value
NGO	Non-governmental organization
PEG	Polyethylene glycol
PEGylated	Polyethylene glycol addition onto molecule of interest
POPC/PC	16:0-18:1 PC 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPE/PE	16:0-18:1 PE 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine

POPG/PG	16:0-18:1 PG 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)
PBS	Phosphate buffered saline
QCM-D	Quartz Crystal Microbalance with Dissipation monitoring
SAMs	Self-assembled monolayers
SBIR	Small business innovation research grant
SDS	Sodium Dodecyl Sulphate
SEM	Scanning electron microscopy
SLB	Supported lipid bilayer
SLIPS	Slippery liquid infused porous surfaces
STEM	Science, technology, engineering, and mathematics
TEM	Transmission electron microscopy

Chaper 1: Specific Aims

1.1 Specific Aims

Rising antibiotic resistance and changes in reimbursement policy brought on by the Affordable Care Act has led to the call for alternative methods to deal with hospital acquired or device acquired infections (HAIs) [1-4]. Central venous catheters (CVCs) and Foley urinary catheters are used in many medical procedures to deliver critical and lifesaving treatments, such as antibiotics, and allow for urinary drainage during long surgeries. However, central line-associated bloodstream infections (CLABSIs) and catheter-associated urinary tract infections (CAUTIs) associated with these devices negatively impact over 550,000 patients per year and cost over $3.5 billion in direct annual cost, according to the Center for Disease Control [2, 3].

Antimicrobial peptides (AMPs) have received commercial interest due to their broad spectrum antimicrobial activity and low likelihood of resistance due to their unique mechanisms of action [4-8]. Few AMPs have made it successfully into clinical practice and none have been approved for non-topical applications [7, 9]. Several drawbacks to AMPs include proteolytic degradation, low salt tolerance, cytotoxicity, and high cost relative to traditional antibiotics [4, 10-13]. Covalently binding or tethering AMPs to a surface has been shown to reduce proteolytic degradation and reduce cytotoxicity without significantly affecting antimicrobial activity [14, 15].

Chrysophsin-1 (CHY1) is a potent salt tolerant AMP which demonstrates excellent activity in solution [16]. CHY1 can be modified N-terminally with cysteine (C-CHY1) without negatively impacting its antimicrobial activity [17, 18]. Despite the potency of C-CHY1 in solution, its antimicrobial activity decreases significantly when tethered by a spacer to a surface, especially compared to other tethered studies where significant AMP activity is maintained [18-25]. This low efficacy is a major hurdle for clinical application of tethered C-CHY1. Thus, the overall goal is to better understand the mechanism of action (MOA) of C-CHY1 in solution as well as the structure-activity relationship of peptide density and spacer length on the antimicrobial activity of tethered C-CHY1. **We hypothesize that there is a relationship between the antimicrobial activity of tethered C-CHY1 and both spacer length and peptide surface density. Peptide**

density may be optimized via reaction temperature and salt concentration, increasing available surface binding sites. Supported lipid bilayers (SLBs) will allow for a better understanding of the MOA leading to better coating design, which will allow feasible development of a clinical business case for tethered AMPs. Toward this end, we propose the following specific aims (SA):

SA1: Characterize the relationship between spacer length and antimicrobial activity of tethered C-CHY1.

Rationale: The addition of the cysteine amino acid did not significantly affect AMP activity however tethering the peptide negatively affected the activity. By tethering the peptide to the surface this effects the ability of the peptide to conform into different secondary and tertiary structures as well as motility [18, 19, 22-27]. It is important that peptide activity is maintained or enhanced in order to have merit as a healthcare solution.

Approach: Use three different length (molecular weight) polyethylene glycol (PEG) spacers to examine the effect of spacer length on activity using the QCM-D which allow for the non-destructive characterization of density and thickness of C-CHY1 layers, which can directly be coupled to antimicrobial activity (against *E. coli* and *S. aureus*) via live-dead staining and fluorescent imaging.

SA2: Determine the effect of bacterial membrane composition and temperature on soluble and bound C-CHY1 activity

Rationale: While cysteine modifications did not significantly alter C-CHY1 activity in solution, the activity of bound C-CHY1 was significantly different. Supported lipid bilayers (SLBs) can be used to elucidate soluble peptide mechanisms of action against model membranes with different compositions, such as Gram-negative and Gram-positive bacteria with lipoteichoic acid (LTA) and lipopolysaccharides (LPS), respectively [28-36]. When bound, peptide density, which is directly related to charge density, is suspected to be an

important parameter leading to altered antimicrobial activity, mechanism of action and biocompatibility [18, 19, 24, 26, 37]. Environmental factors, such as salt concentrations and temperature changes may lead to significant changes in bound C-CHY1 density, and thus, mechanisms [38-41].

SA2.1: Study the influence of reaction conditions on binding efficiency and activity of C-CHY1.

Approach: Use the QCM-D to study the interactions of C-CHY1 with model SLBs to examine the effects of temperature (23C and 37C), peptide concentration (5uM), and lipid composition. Two and three component lipid bilayer systems representative of Gram-negative and Gram-positive bacteria will be formed. POPC/POPG with a 4:1 molar ratio and POPC/POPG/LTA with a 4:1 molar to 0.1 weight ratio of LTA will be used to represent Gram-positive bacteria. POPE/POPG with a 4:1 molar ratio and POPE/POPG/LPS with a 4:1 molar to 0.1 weight ratio of LPS will be used to represent Gram-negative bacteria. A weight ratio was used for the LTA and LPS because no molecular weight was readily available as they are components purified from living cells.

SA3: Demonstrate how entrepreneurial models can be taught and applied to domestic and international business models for commercializing AMP technologies.

Rationale: Novel medical device technologies have the potential to solve many unmet needs. However, these discoveries are not useful unless they can be commercialized. An entrepreneurial mindset is needed in order for researchers to identify the biggest needs, and tailor their solutions to these issues. In particular, innovation is desperately needed to combat antibiotic resistance in developing nations, which will be disproportionately affected by this threat [42]. Commercialization is particularly difficult in underdeveloped nations. Our goal was to understand how innovations have been introduced into developing nations, where they have failed, and where things went well [43]. We examine one graduate program's attempt at a potentially transformative program to instill an entrepreneurial mindset in STEM Ph.D. students [44]. Entrepreneurial solutions from academia are needed to tackle some of the largest global challenges, including the challenge of antimicrobial resistance.

Approach: WPI has a world class project based learning curriculum where students work on projects provided by sponsors globally, known as the Interactive Qualifying Project (IQP), where students use their technical expertise to contribute to solving society's biggest challenges. We examined an IQP as a case study in order to learn best practices in implementing technological advances into local communities [43]. We used data collected through WPI's NSF-funded PhD training program, *IGERT: Training Innovative Leaders in Biofabrication*, in order to examine the best ways to instill an entrepreneurial mindset in graduate students [44]. We used the business model canvas, developed by Alex Osterwalder, in order to develop a feasible and scalable business plan that addresses the steps to take the product, an antimicrobial surface coating, to market [45]. We fostered relationships with experts and mentors with connections to the urinary catheter market and several other medical device industries to test our hypotheses [46-48]. These relationships will build on our finding from research and the NSF I-Corps experience, which is designed to examine the potential economic and societal benefits of NSF funded research.

References

1. Centers for Disease Control and Prevention, *Antibiotic resistance threats in the United States, 2013. Atlanta: CDC; 2013*. 2014.
2. Scott, R.D., *The direct medical costs of healthcare-associated infections in US hospitals and the benefits of prevention.* 2009.
3. Centers for Disease Control and Prevention. *State-Specific Supplement to the National Healthcare-Associated Infection Standardized Infection Ratio Report: July 2009 through December 2009*. 2009 March 2, 2011 [cited 2019 January 28]; Available from: https://www.cdc.gov/HAI/surveillance/reports/state-specific-hai-sir-luly-dec-2009.html.
4. Li, P., et al., *Antimicrobial macromolecules: synthesis methods and future applications.* Rsc Advances, 2012. **2**(10): p. 4031-4044.
5. Jenssen, H., P. Hamill, and R.E.J.C.m.r. Hancock, *Peptide antimicrobial agents.* 2006. **19**(3): p. 491-511.

6. Nizet, V., *Antimicrobial peptide resistance mechanisms of human bacterial pathogens.* Current issues in molecular biology, 2006. **8**(1): p. 11.

7. Marr, A.K., W.J. Gooderham, and R.E. Hancock, *Antibacterial peptides for therapeutic use: obstacles and realistic outlook.* Current opinion in pharmacology, 2006. **6**(5): p. 468-472.

8. Reddy, K., R. Yedery, and C. Aranha, *Antimicrobial peptides: premises and promises.* International journal of antimicrobial agents, 2004. **24**(6): p. 536-547.

9. Salwiczek, M., et al., *Emerging rules for effective antimicrobial coatings.* Trends in Biotechnology, 2014. **32**(2): p. 82-90.

10. Brogden, N.K. and K.A. Brogden, *Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals?* International journal of antimicrobial agents, 2011. **38**(3): p. 217-225.

11. Batoni, G., et al., *Use of antimicrobial peptides against microbial biofilms: advantages and limits.* Current medicinal chemistry, 2011. **18**(2): p. 256-279.

12. Khoo, X. and M.W. Grinstaff, *Novel infection-resistant surface coatings: a bioengineering approach.* Mrs Bulletin, 2011. **36**(5): p. 357-366.

13. Eckert, R., *Road to clinical efficacy: challenges and novel strategies for antimicrobial peptide development.* Future microbiology, 2011. **6**(6): p. 635-651.

14. Hickok, N.J. and I.M. Shapiro, *Immobilized antibiotics to prevent orthopaedic implant infections.* Advanced drug delivery reviews, 2012. **64**(12): p. 1165-1176.

15. Fosgerau, K. and T. Hoffmann, *Peptide therapeutics: current status and future directions.* Drug discovery today, 2015. **20**(1): p. 122-128.

16. Iijima, N., et al., *Purification and characterization of three isoforms of chrysophsin, a novel antimicrobial peptide in the gills of the red sea bream, Chrysophrys major.* European Journal of Biochemistry, 2003. **270**(4): p. 675-686.

17. Ivanov, I.E., et al., *Creating Antibacterial Surfaces with the Peptide Chrysophsin-1*. ACS Applied Materials & Interfaces, 2012. **4**(11): p. 5891-5897.

18. Lozeau, L.D., T.E. Alexander, and T.A. Camesano, *Proposed mechanisms of tethered antimicrobial peptide chrysophsin-1 as a function of tether length using QCM-D.* The Journal of Physical Chemistry B, 2015. **119**(41): p. 13142-13151.

19. Li, Y., et al., *Effects of peptide immobilization sites on the structure and activity of surface-tethered antimicrobial peptides.* The Journal of Physical Chemistry C, 2015. **119**(13): p. 7146-7155.

20. Li, X., et al., *Antimicrobial functionalization of silicone surfaces with engineered short peptides having broad spectrum antimicrobial and salt-resistant properties.* Acta biomaterialia, 2014. **10**(1): p. 258-266.

21. Yu, K., et al., *Anti-adhesive antimicrobial peptide coating prevents catheter associated infection in a mouse urinary infection model.* Biomaterials, 2017. **116**: p. 69-81.

22. Haynie, S.L., G.A. Crum, and B.A. Doele, *Antimicrobial activities of amphiphilic peptides covalently bonded to a water-insoluble resin.* Antimicrobial Agents and Chemotherapy, 1995. **39**(2): p. 301-307.

23. Costa, F., et al., *Covalent immobilization of antimicrobial peptides (AMPs) onto biomaterial surfaces.* Acta biomaterialia, 2011. **7**(4): p. 1431-1440.

24. Bagheri, M., *Cationic Antimicrobial Peptides: Thermodynamic Characterization of Peptide-Lipid Interactions and Biological Efficacy of Surface-Tethered Peptides*. 2010, Freie Universität Berlin.

25. Onaizi, S.A. and S.S. Leong, *Tethering antimicrobial peptides: current status and potential challenges.* Biotechnology advances, 2011. **29**(1): p. 67-74.

26. Hilpert, K., et al., *Screening and characterization of surface-tethered cationic peptides for antimicrobial activity.* Chemistry & biology, 2009. **16**(1): p. 58-69.

27. Bagheri, M., M. Beyermann, and M. Dathe, *Immobilization reduces the activity of surface-bound cationic antimicrobial peptides with no influence upon the activity spectrum.* Antimicrobial agents and chemotherapy, 2009. **53**(3): p. 1132-1141.

28. Wang, Y., R. Narain, and Y. Liu, *Study of Bacterial Adhesion on Different Glycopolymer Surfaces by Quartz Crystal Microbalance with Dissipation.* Langmuir, 2014. **30**(25): p. 7377-7387.

29. Wang, K.F., et al., *Characterization of supported lipid bilayer disruption by chrysophsin-3 using QCM-D.* The Journal of Physical Chemistry B, 2011. **115**(51): p. 15228-15235.

30. Wang, K.F., R. Nagarajan, and T.A. Camesano, *Differentiating antimicrobial peptides interacting with lipid bilayer: molecular signatures derived from quartz crystal microbalance with dissipation monitoring.* Biophysical chemistry, 2015. **196**: p. 53-67.

31. Lozeau, L.D., et al., *A QCM-D study of the concentration-and time-dependent interactions of human LL37 with model mammalian lipid bilayers.* 2018. **167**: p. 229-238.

32. Kamaloo, E., *Supported lipid bilayer interactions with nanoparticles, peptides and polymers.* 2018.

33. Michel, J.-P., et al., *Disruption of Asymmetric Lipid Bilayer Models Mimicking the Outer Membrane of Gram-Negative Bacteria by an Active Plasticin.* Langmuir, 2017. **33**(41): p. 11028-11039.

34. Malanovic, N. and K. Lohner, *Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2016. **1858**(5): p. 936-946.

35. Brown, S., J.P. Santa Maria, Jr., and S. Walker, *Wall teichoic acids of gram-positive bacteria.* Annual review of microbiology, 2013. **67**: p. 313-336.

36. Hardy, G.J., R. Nayak, and S. Zauscher, *Model cell membranes: Techniques to form complex biomimetic supported lipid bilayers via vesicle fusion.* Current opinion in colloid & interface science, 2013. **18**(5): p. 448-458.

37. Kügler, R., O. Bouloussa, and F. Rondelez, *Evidence of a charge-density threshold for optimum efficiency of biocidal cationic surfaces.* Microbiology, 2005. **151**(5): p. 1341-1348.

38. Torcato, I.M., et al., *Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2013. **1828**(3): p. 944-955.

39. Han, H.M., R. Gopal, and Y. Park, *Design and membrane-disruption mechanism of charge-enriched AMPs exhibiting cell selectivity, high-salt resistance, and anti-biofilm properties.* Amino Acids, 2016. **48**(2): p. 505-522.

40. Deslouches, B., et al., *Rational design of engineered cationic antimicrobial peptides consisting exclusively of arginine and tryptophan, and their activity against multidrug-resistant pathogens.* Antimicrobial agents and chemotherapy, 2013. **57**(6): p. 2511-2521.

41. Chen, Y., et al., *Rational design of α-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index.* Journal of biological chemistry, 2005. **280**(13): p. 12316-12329.

42. O'Neill, J., *Antimicrobial resistance: tackling a crisis for the health and wealth of nations.* Rev. Antimicrob. Resist, 2014. **20**: p. 1-16.

43. Dana, L.-P., et al., *Success factors and challenges of grassroots innovations: Learning from failure*. 2019.

44. Alexander, T.E., Lozeau, Linsday D., Camesano, Terri A., Hoy, Frank, *Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program.* Portland International Center for Management of Engineering and Technology (PICMET), 2019.

45. Osterwalder, A. and Y. Pigneur, *Business model generation: a handbook for visionaries, game changers, and challengers*. 2010: John Wiley & Sons.

46. Blank, S. and B. Dorf, *The startup owner's manual: The step-by-step guide for building a great company*. 2012: BookBaby.

47. Blank, S., *What's A Startup? First Principles.* 2010: https://steveblank.com/.

48. Blank, S., *Why the lean start-up changes everything.* Harvard business review, 2013. **91**(5): p. 63-72.

Chapter 2 Background

2.1 Background

Antimicrobial resistance is a growing global threat which will kill 10 million people annually by 2050 with a total economic burden of $100 trillion if nothing is done to stop it [1]. In the US, the economic burden associated with healthcare associated infections is estimated to cost $45 billion annually in direct medical costs [2]. There are an estimated 2 million reported antimicrobial resistant infections in the US a year which leads to 23,000 deaths [3]. Unfortunately there has not been significant development or approval of new classes of antibiotics which is exacerbated by a rise in multidrug resistant bacteria threatening the usefulness of last resort antibiotics [3]. In between 1980 and 1984, 18 new antibiotics were approved by the FDA, but this has declined ever since. In fact, between 2010 and 2012, only 2 new antibiotics were approved [3]. In order to try to combat the drying antibiotic pipeline the Generating Antibiotic Incentives Now (GAIN) Act was passed in 2012, which incentivizes antimicrobial research and development by extending patent life protection for new antibiotics as well as providing priority and possibly fast track review through the FDA [4]. Between 2015 and 2017, there were 6 new antimicrobials approved; however, half of these new drugs had very narrow applications, two were for new combinations of previously known antibiotics, and one was a derivative of fluoroquinolones [5]. No novel classes of antimicrobials were introduced which highlights the need for innovation in this space [5, 6].

Many of these infections are associated with medical devices such as orthopedic knee and hip replacements, stents, central venous catheters (CVCs), and Foley urinary catheters [2, 3, 7, 8]. In fact, Foley urinary catheters are the number one cause of hospital acquired infections, known as catheter associated urinary tract infections (CAUTIs) [2, 9]. The cost to treat CAUTIs is $1 billion annually in the US, which equals the market size of Foley catheters [9, 10]. These infections cause increased patient pain and discomfort increasing patient length of stay (LOS) and increased morbidity [2, 9-11]. Additionally these infections are known as *never events*, defined as an "event that never should have occurred in the first place", and are non-reimbursable by Medicare and Medicaid which costs hospitals approximately $1000 per case [12]. Bottom-

performing hospitals can also be penalized by another 2% of their Medicare and Medicaid budgets, which considerably adds to the financial burden.

Medical device associated infections require the use of traditional antibiotics for treatment, which increases the occurrence of antibiotic resistance in bacteria [12]. One in four HAIs are associated with resistant bacteria [13]. When on surfaces, of medical devices, bacteria are often in the form of a biofilm. Biofilms are communities of bacteria that produce an extracellular matrix, which makes the bacteria up to 1000 times more resistant to antibiotics than planktonic bacteria (e.g. in solution) and in turn increases the likelihood of propagating additional resistant strains [14-19]. Current alternatives to treatment with antibiotics, such as antimicrobial-impregnated catheters may promote bacterial resistance, are cytotoxic and are geno-toxic [20-27]. Infections associated with resistant bacteria are twice as expensive to treat compared to their antibiotic sensitive counterparts and mortality it doubled with resistant strains [13]. Treatments that do not promote bacterial resistance, while having the ability to kill resistant bacteria such as MRSA are desperately needed [2, 3, 11]. Further, treatments that prevent infection in the first place are ideal [2, 3, 13, 16, 28].

Antimicrobial peptides (AMPs) are a promising alternative to traditional antibiotics. AMPs are proteins that are part of the innate immunity of a wide variety of species, and exert antimicrobial activities via several different mechanisms of action compared to traditional antibiotics [29-32]. Typically, AMPs are 10-50 amino acids, amphipathic (having hydrophilic and hydrophobic residues) and cationic (+2 to +9) [29-32]. AMPs have broad spectrum activity against many types of microbes, and a low likelihood of promoting antimicrobial resistance due to their unique mechanisms of action. These mechanisms often involve non-specific interactions with bacterial membranes leading to the formation of transient or permanent membrane pores which leads to loss of homeostasis and cell death [6, 29-32]. Some peptides have intracellular targets and some peptides even have concentration-dependent mechanisms of action [32-36]. Additionally some AMPs, such as human-derived LL-37, influence the inflammatory response and even wound healing [37, 38].

Despite the promise of AMPs for clinical implementation, none have been approved thus far for clinical use [39]. This is due to a number of reasons including AMP toxicity, cost of production, low half-life *in vivo*, and a lack of comprehensive knowledge of individual peptide mechanisms of action [32, 39-44]. Many AMPs initially interact with bacteria via electrostatic interactions, which can be diminished or eradicated in high salt environments such as blood serum or urine [34, 45-47]. Further, some mechanisms of action for AMP resistance, although rare, are implemented by bacteria. These include alterations to membrane surface structure that reduce overall charge, creation of efflux pumps and production of proteases [48, 49]. Due to these issues, most current AMPs in the FDA pipeline are for topical applications, which not only limits their breadth of functionality, but also their ability to combat antimicrobial resistance [50].

Many different strategies have been adopted to overcome the drawbacks of AMPs while maintaining or enhancing their antimicrobial activity [6, 30, 34, 41, 47, 50-58]. Several strategies to reduce AMP cytotoxicity have been implemented [34, 42, 59]. This includes alteration to the primary amino acid sequence of the AMPs in the form of truncation, addition or substitutions and in the inclusion of unusual amino acids [54, 60-66]. The cytotoxic termini of some AMPs have been PEGylated, and even tethered onto surfaces to alleviate cytotoxicity [6, 37, 42, 55, 67-69]. Unfortunately, changes to the primary sequence often leads to changes in the secondary and tertiary structures of AMPs, which can have a negative impact on mechanism and antimicrobial activity [6, 60, 70]. The cost of AMP production is often high, on average 1000/g or more depending upon peptide length and amino acid makeup [39]. Zasloff [39], was able to achieve a cost of $100/g, but this took a significant effort to achieve and the method is undisclosed. In order for efficient scale-up and production for clinical implementation, recombinant methods of production have been investigated, though very little is known about these processes and they must be optimized for each peptide sequence [42, 58, 71-73]. Attempts to extend the *in vivo* half-life and reduce proteolytic degradation of AMPs by making D-amino acid substitutions, inducing peptide cyclization, slow release systems, and adsorption or binding of AMPs onto surfaces have been examined [6, 42, 43, 60, 66, 74-76]. Many of the issues prohibiting AMPs from

clinical implementation could be addressed with rational design of synthetic versions of AMPs [40, 41, 51, 52, 54, 63, 66]. These designs could be informed by a detailed understanding of individual peptide mechanisms of action; however, there are many gaps in this area stemming from a lack of techniques available to study AMP mechanism [6, 33, 43, 77-81]. The development of techniques that can study real-time interactions of peptides and their analogues against bacterial and mammalian cells would be critical to further development of less toxic peptides with improved antimicrobial action [6, 33, 37, 78, 82]. Improving upon all AMP shortcomings with one overarching solution has proven to be difficult [32, 41-43, 48, 50, 56, 60, 77, 79].

One strategy that may help overcome these issues is covalently tethering (or, binding) AMPs onto a surface such as a biomaterial or medical device, in order to prevent infection [6, 8, 43, 55, 57, 83]. By tethering the peptide proteolytic degradation (protection from proteases), cytotoxicity (only interaction with bacteria), and cost can be reduced by localizing the peptide to where it is needed creating a contact active surface [6, 32, 35, 80]. There have been many methods implemented to tether (via a "spacer") AMPs onto the surface [6, 37, 43, 44, 84]. There is disagreement in the literature on how the many tethering parameters, including spacer length, peptide surface density, tether flexibility and tethering chemistry effect the activity of AMPs [43, 55, 57, 83, 85-88]. Further complicating the study of tethering is that there are few techniques that can study all tethering parameters at once [6, 43, 44, 57, 85, 89]. To adequately study peptide tethering first requires detailed understanding of their soluble mechanisms of action [78, 90, 91]. Current studies employs multiple, often destructive, techniques, that are not in real-time, and do not always allow for the direct coupling of antimicrobial activity to the physical parameter data collected [6, 43, 57].

The quartz crystal microbalance with dissipation monitoring (QCM-D) is a non-destructive acoustic technique that measures the changes in mass and rigidity of a nanoscale-sized film in real time [6, 82, 92]. Further, it allows determination of film thickness and density non-destructively, and then can be directly coupled to antimicrobial activity via other techniques [6, 82]. QCM-D has been used to study concentration-,

temperature- and lipid composition-, and environment-dependent interactions of peptides against model cell membranes of red blood cells, other mammalian cells, and bacterial cells [78, 82, 90-92]. QCM-D data can be analyzed in several different ways to gather detailed information about specific peptide mechanism, which can be related to *in vitro* cytotoxicity, antimicrobial activity, or both [6, 78, 82, 90, 91]. Further, QCM-D has been used to monitor real-time tethering processes of AMPs, such as the zero-length cysteine-coupling of porcine-derived Cecropin P1 onto gold surfaces [93], the direct adsorption of LL37 onto silicon dioxide [37, 78], and even the attachment of marine Chrysophsin-1 (CHY1) onto poly(ethylene glycol) (PEG) tethers [94]. The QCM-D allows for buildup and thickness measurements of the surface coating, and then the exposure of peptide surfaces to different environmental conditions such as temperature or changes in salt concentrations, and even the introduction of bacteria to monitor antimicrobial activity [6, 33, 37, 78, 82, 90, 91].

Early studies by Ivanov *et al.* [94] demonstrated that the marine-derived AMP Chrysophsin-1 (CHY1) could be bound to a surface and maintain antimicrobial activity when tethered via a PEG tether. CHY1 in a highly cationic, 25-amino acid, α-helical AMP found in the gills of *Chrysophrys major* (red sea bream) with broad spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria [95]. It possesses naturally high salt tolerance because of its marine derivation, which makes it attractive to investigate as a potential AMP-based therapeutic, but unfortunately it is hemolytic at relatively low concentrations compared to its antimicrobial concentrations [95]. In order to utilize this AMP effectively to prevent device associated infections CHY1 peptide was modified with a cysteine residue at the N-terminal end to form a 26-amino acid derivative AMP called C-CHY1, which would to allow for covalent tethering, onto a surface. We hypothesized that this strategy would to reduce the cytotoxicity of C-CHY1 while maintaining its antimicrobial activity [94]. C-CHY1 was covalently bound to a surface using a hetero bi-functionalized polyethylene glycol (PEG) spacer using a maleimide chemistry [94]. However, Ivanov *et al.* [94] only examined one tether (spacer) length. Spacer, or tether, length is an important parameter that significantly effects bound AMP activity [43, 44, 55, 84]. Thus, we related PEG tether length to C-CHY1 antimicrobial activity of the surface using three

different PEG lengths using QCM-D, which informed rational design decisions regarding PEG length [6]. Density is also an important parameter for tethered AMP activity which was measured but not optimized for when studying the effect of spacer length [6, 43, 44, 55, 69, 84, 87]. Therefore we studied the effect of AMP density by altering the amount of PEG binding on the surface using temperature and salt concentration [6, 43, 44, 84, 96, 97]. It has been shown that even a one amino acid addition, substitution, or removal can have a significant effect on activity and on mechanism of action of the peptide [30-32, 34-36, 39-42, 47, 50, 51, 58, 60, 74, 84, 98-103]. Thus, we examined the mechanism of action using complex supported lipid bilayers [78, 90, 91, 104-106]. This mechanism of action is important to understand in order to rationally design antimicrobial coatings to prevent infections [6, 32, 42-44, 48, 50].

This thesis additionally addresses a business case for tethered C-CHY1 clinical implementation. Novel antimicrobials are needed to address the growing problem of antimicrobial resistance; this requires not only discovery but commercialization [1, 107]. Over 10 million people a year, more people a year than cancer, will die due to antimicrobial resistance unless new treatments are developed [1]. This will disproportionally effect countries in Africa and Asia, **Figure 1**. It is not just enough to develop new therapies in the laboratory setting, but there must be a push to translate these ideas into innovations and get them into the market place.

Figure 1: Number of death caused by antimicrobial resistance per year in six global regions. Reprinted under the Creative Commons Attribution 4.0 International Public License. Attribution notice: Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. 2014.

Academic institution involvement in this space is needed due to the fact that the net present value (NPV) to develop novel antimicrobials for pharmaceutical companies is negative [107, 108]. Besides the net negative NPV the cost to take a new drug to market is approximately $1.4 billion requiring a large allocation of resources and high risk [109].This means that the burden for development falls on universities and small companies which do not have the resources of the larger companies but could help lower pre-human trial cost which account for 30% of drug development [109]. Additionally there are programs and incentives for early stage start-ups and small companies to develop novel antimicrobials [107]. These incentives are lacking for

larger companies [107]. However only ~5% of university patents are ever licensed [110]. One of the major hurdles is that often times the Ph.D. chemists, biologist, and engineers that are likely to come up with the solutions are not trained to take their ideas to market [111-113]. One solution is to train science and engineering Ph.D. students in the field of entrepreneurship to help instill an entrepreneurial mindset [113]. This does not mean that every Ph.D. student will start their own company but this entrepreneurial mindset has other benefits for the students as well such as improved communication skills (ability to talk to a non-technical audience), clarity in career path and comfortability working in interdisciplinary teams [112, 113]. Such programs have successfully been integrated into Ph.D. programs with highly positive outcomes for the students that were involved, **Figure 2** [111-113].

Figure 2*:* Measured outcomes of 20 Ph.D. students in the WPI IGERT program. Reprinted with permission from Alexander, T.E., Lozeau, Linsday D., Camesano, Terri A., Hoy, Frank, Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program. Portland International Center for Management of Engineering and Technology (PICMET), 2019.

Even with entrepreneurship training many Ph.D. students will find it difficult to put together a traditional business plan, however it is still an important to develop a business plan [114, 115]. A formal business plan alternative that has grown in popularity due to its simplicity as well as ease of visualization is

The Business Model Canvas (BMC), **Figure 3**. Alexander Osterwalder developed the BMC following the Lean Start-Up methodology [114, 115]. The BMC contains all the elements of a traditional business model but is more adaptable, allowing for pivots to be made quickly and easily and the flexibility required of an early stage start up [115]. The business model canvas can be divided into two major sections, the right side of the canvas is focused in the customer, and the left side focuses on the business [114-116]. Assumptions (hypotheses) are made and placed in the business model canvas [114-116]. These assumptions are tested by talking to experts and customers, if an assumption is confirmed then it remains on the canvas, if it is rejected a new hypothesis is added based on the new information then tested [114-116]. An iterative process is then continued until all the hypothesis are confirmed and there are no unanswered questions, at this point a scalable business model has been developed [114-116].

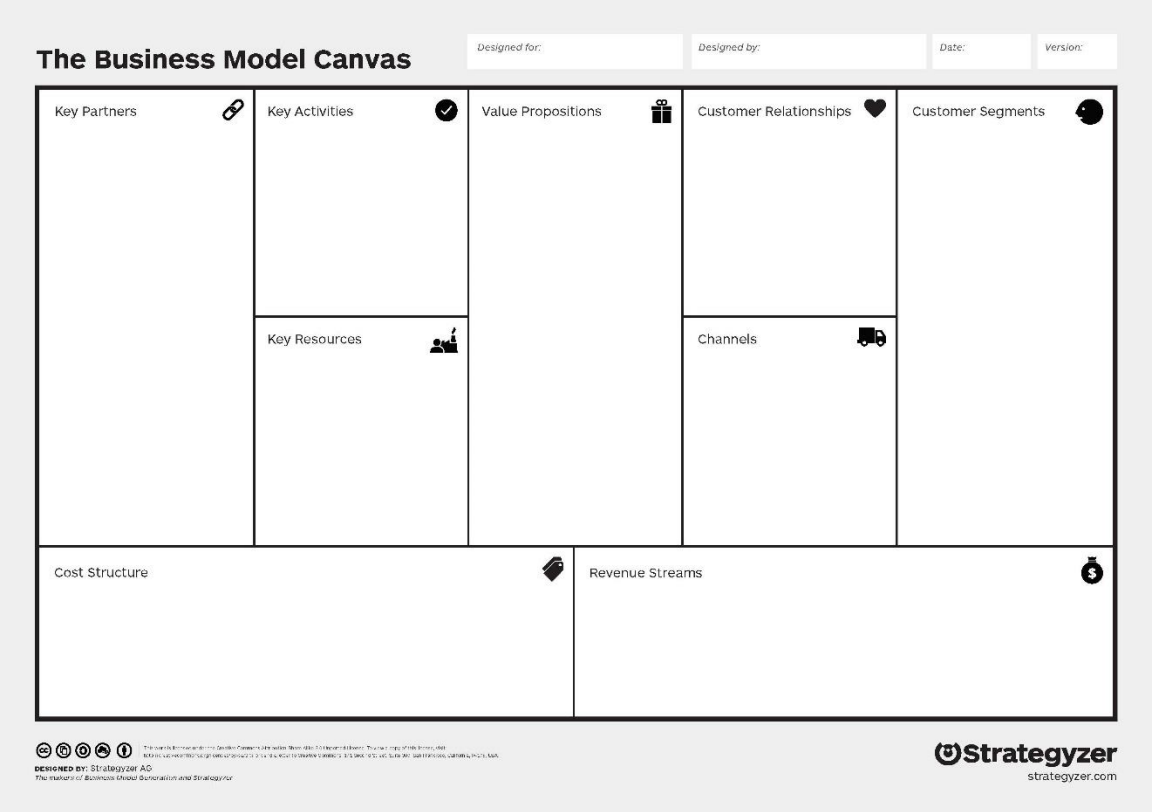


Figure 3: The Business Model Canvas as developed by Alexander Osterwalder. It allows for a quick visualization of all the key elements of a business plan. This allows for rapid iteration as well as visual representation of the gaps that need to be filled.

The BMC allows for the formation and testing of hypotheses and quick iterations that are important to developing a scalable, sustainable business model [115-117]. The Innovation Corps (I-Corps) program at the National Science Foundation (NSF) for startups and commercial ideas relieves heavily on the BMC and lean startup methodology. While only 14% of researchers who apply for a small business innovation research (SBIR) grant receive funding, 60% of applicants who went through the I-Corps program receive SBIR funding [118]. Even though the BMC is a relatively new model, it has proven to be successful and therefore a business model canvas was developed for this project with results that can be extended to other AMP based products. In order to test hypothesis, in each of the BMC categories, and iterate on the BMC we performed over 250 customer and expert interviews, including 100 during the NSF I-Corps program [119].

Commercialization of medical devices is significantly different from country to country with many different regulatory bodies and varying levels of regulations [120, 121]. As seen in Figure 1 many of the countries that will be most negatively impacted by antimicrobial resistance are in Africa. Just like any other difference from country to country, no two African countries have the same regulatory system and infrastructure. However, for the purposes of our study, we selected Nigeria as an example [122]. Nigeria was selected because it is the largest country in Africa by population, there is an established regulatory system (with potential regulatory synergy with the EU), and a large number of Worcester Polytechnic Institute (WPI) Interactive Qualifying Projects (IQPs) were available to be used as case studies [122, 123].

We identified a WPI IQP, Woodyard *et al.* [124], project that fit 3 important criteria:

1) it involved a new innovative product,

2) the product had failed, and

3) lessons learned from the previous failure were applied and the product was relaunched

The product itself was not medical device related, it was a dense paper block meant to be used as fuel for cooking food, however it offered many transferable lessons learned [124, 125]. In their study the IQP group found that the Men on the Side of the Road (MSR, a non-profit) came up with a solution to a problem

but did not fully understand their customers' problems which led to failure. This highlights the importance of the core principles of the lean methodology: understanding who your customer is and listening to what they have to say [114, 116, 118]. Similarly a product may meet the medical need but may not fit into the treatment regimen for a number of reasons, for example need for training [124]. These issues echo those faced by the medical device industry when introducing new products into these markets [126, 127]. It is important that when looking into launching a product into a country like Nigeria other factors in the healthcare landscape are understood [122, 128, 129]. In our case if we can significantly reduce the infection of medical devices there is less of a need to use traditionalantibiotics which are often unavailable, leading to better patient outcomes [2, 28, 128-130].

Antimicrobial resistance is an emergent global problem that requires innovative solutions be brought to market [1, 3, 13, 50]. The goal of this research was to investigate the parameters of tether length and density on the covalently bound AMP C-CHY1, and to understand its mechanism of action in order to rationally design an antimicrobial coating to prevent infections of medical devices. We developed a pathway towards commercialization for this peptide that can be used as a roadmap for other AMP based products. We also studied how entrepreneurship education can improve the graduate student experience and how that may lead to an increase in the commercialization of beneficial technologies.

References

1. O'Neill, J., *Antimicrobial resistance: tackling a crisis for the health and wealth of nations.* Rev. Antimicrob. Resist, 2014. **20**: p. 1-16.
2. Scott, R.D., *The direct medical costs of healthcare-associated infections in US hospitals and the benefits of prevention.* 2009.
3. Centers for Disease Control and Prevention, *Antibiotic resistance threats in the United States, 2013. Atlanta: CDC; 2013*. 2014.
4. Gingrey, P., *Generating Antibiotic Incentives Now Act of 2011*, H.a.H. Services, Editor. 2012, Department of Health and Human Services
5. Andrei, S., et al., *New FDA approved antibacterial drugs: 2015-2107*. 2018, Discoveries.
6. Lozeau, L.D., T.E. Alexander, and T.A. Camesano, *Proposed mechanisms of tethered antimicrobial peptide chrysophsin-1 as a function of tether length using QCM-D.* The Journal of Physical Chemistry B, 2015. **119**(41): p. 13142-13151.
7. Darouiche, R.O., *Treatment of infections associated with surgical implants.* New England Journal of Medicine, 2004. **350**(14): p. 1422-1429.
8. Hickok, N.J. and I.M. Shapiro, *Immobilized antibiotics to prevent orthopaedic implant infections.* Advanced drug delivery reviews, 2012. **64**(12): p. 1165-1176.
9. Sullivan, F., *U. S. Urinary Incontinence Devices*, F. Sullivan, Editor. 2011.
10. Lehr, P., *Global Markets for Catheters A BCC Research Report*, B. Research, Editor. 2012: BCC Research.
11. Pickard, R., et al., *Antimicrobial catheters for reduction of symptomatic urinary tract infection in adults requiring short-term catheterisation in hospital: a multicentre randomised controlled trial.* The lancet, 2012. **380**(9857): p. 1927-1935.
12. Kennedy, E.H., M.T. Greene, and S.J.J.o.h.m. Saint, *Estimating hospital costs of catheter-associated urinary tract infection.* 2013. **8**(9): p. 519-522.

13. O'Neill, J., *Infection prevention, control and surveillance: limiting the development and spread of drug resistance. The review on antimicrobial resistance*. 2016.

14. Atefyekta, S., et al., *Antibiofilm Elastin-like Polypeptide Coatings-Functionality, Stability, and Selectivity.* Acta Biomaterialia, 2018.

15. Chen, M., Q. Yu, and H. Sun, *Novel strategies for the prevention and treatment of biofilm related infections.* International journal of molecular sciences, 2013. **14**(9): p. 18488-18501.

16. Kostakioti, M., M. Hadjifrangiskou, and S.J. Hultgren, *Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era.* Cold Spring Harbor perspectives in medicine, 2013. **3**(4): p. a010306-a010306.

17. Römling, U., et al., *Microbial biofilm formation: a need to act.* Journal of internal medicine, 2014. **276**(2): p. 98-110.

18. Flemming, H.-C. and J. Wingender, *The biofilm matrix.* Nature Reviews Microbiology, 2010. **8**: p. 623.

19. Flemming, H.-C., et al., *Biofilms: an emergent form of bacterial life.* Nature Reviews Microbiology, 2016. **14**: p. 563.

20. ARROW International, *Product Manual: Chloragard Technology Information, 2010*. 2010: http://www.teleflex.com/en/usa/productAreas/vascularAccess/productGroups/arrowErgoPack/safety-information/documents/chloragard-tech-sheet.pdf.

21. COOK Medical, *Product Manual: Evidence-Based Medicine: A Compendium of Studies*. 2012: https://www.cookmedical.com/data/resources/productReferences/IRCC-BM-SABD-EN-201209-interactive_WEB.pdf.

22. Bard Medical Division, *Addressing Antibiotic Resistance: Reducing Antibiotic Use by Avoiding Catheter-Associated Infections IC Proven Results*. 2004: http://www.bardmedical.com/media/3012/PelvicAntibioticRes.pdf.

23. Eady, E., M. Gloor, and J.J.D. Leyden, *Propionibacterium acnes resistance: a worldwide problem.* 2003. **206**(1): p. 54-56.

24. Long, J.W., *Essential Guide to Prescription Drugs 1993*. 1992: HarperCollins Publishers.

25. Lee, T.H., et al., *Cytotoxicity of chlorhexidine on human osteoblastic cells is related to intracellular glutathione levels.* 2010. **43**(5): p. 430-435.

26. Li, Y.C., et al., *Cytotoxicity and genotoxicity of chlorhexidine on macrophages in vitro.* 2014. **29**(4): p. 452-458.

27. Monzillo, V., et al., *Chlorhexidine-silver sulfadiazine-impregnated central venous catheters: in vitro antibacterial activity and impact on bacterial adhesion.* 2012. **35**(2): p. 175.

28. Control, C.f.D. and Prevention, *Urinary tract infection (catheter-associated urinary tract infection [CAUTI] and non-catheter-associated urinary tract infection [UTI]) and other urinary system infection [USI]) events.* Centers for Disease Control and Prevention, Atlanta, GA: http://www. cdc. gov/nhsn/PDFs/pscManual/7pscCAUTIcurrent. pdf, 2015.

29. Hancock, R.E. and G.J.T.i.m. Diamond, *The role of cationic antimicrobial peptides in innate host defences.* 2000. **8**(9): p. 402-410.

30. Hancock, R.E. and R.J.T.i.b. Lehrer, *Cationic peptides: a new source of antibiotics.* 1998. **16**(2): p. 82-88.

31. Hancock, R.E.J.T.L.i.d., *Cationic peptides: effectors in innate immunity and novel antimicrobials.* 2001. **1**(3): p. 156-164.

32. Jenssen, H., P. Hamill, and R.E.J.C.m.r. Hancock, *Peptide antimicrobial agents.* 2006. **19**(3): p. 491-511.

33. Lozeau, L.D., et al., *A QCM-D study of the concentration-and time-dependent interactions of human LL37 with model mammalian lipid bilayers.* 2018. **167**: p. 229-238.

34. Marr, A.K., W.J. Gooderham, and R.E. Hancock, *Antibacterial peptides for therapeutic use: obstacles and realistic outlook.* Current opinion in pharmacology, 2006. **6**(5): p. 468-472.

35. Brogden, K.A., *Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?* Nature reviews microbiology, 2005. **3**(3): p. 238.

36. Zasloff, M., *Antimicrobial peptides of multicellular organisms.* nature, 2002. **415**(6870): p. 389.

37. Lozeau, L.D., et al., *Collagen tethering of synthetic human antimicrobial peptides cathelicidin LL37 and its effects on antimicrobial activity and cytotoxicity.* 2017. **52**: p. 9-20.

38. Carretero, M., et al., *In vitro and in vivo wound healing-promoting activities of human cathelicidin LL-37.* Journal of Investigative Dermatology, 2008. **128**(1): p. 223-236.

39. Zasloff, M., *Antimicrobial Peptides: Do They Have a Future as Therapeutics?* 2016. p. 147-154.

40. Reddy, K., R. Yedery, and C. Aranha, *Antimicrobial peptides: premises and promises.* International journal of antimicrobial agents, 2004. **24**(6): p. 536-547.

41. Mishra, B., et al., *Host defense antimicrobial peptides as antibiotics: design and application strategies.* Current Opinion in Chemical Biology, 2017. **38**: p. 87-96.

42. Eckert, R., *Road to clinical efficacy: challenges and novel strategies for antimicrobial peptide development.* Future microbiology, 2011. **6**(6): p. 635-651.

43. Onaizi, S.A. and S.S. Leong, *Tethering antimicrobial peptides: current status and potential challenges.* Biotechnology advances, 2011. **29**(1): p. 67-74.

44. Hilpert, K., et al., *Screening and characterization of surface-tethered cationic peptides for antimicrobial activity.* Chemistry & biology, 2009. **16**(1): p. 58-69.

45. Bowdish, D.M., et al., *Impact of LL-37 on anti-infective immunity.* Journal of leukocyte biology, 2005. **77**(4): p. 451-459.

46. Tam, J.P., Y.-A. Lu, and J.-L. Yang, *Correlations of cationic charges with salt sensitivity and microbial specificity of cystine-stabilized β-strand antimicrobial peptides.* Journal of Biological Chemistry, 2002. **277**(52): p. 50450-50456.

47. Chu, H.-L., et al., *Boosting Salt Resistance of Short Antimicrobial Peptides.* Antimicrobial Agents and Chemotherapy, 2013. **57**(8): p. 4050.

48. Andersson, D.I., D. Hughes, and J.Z. Kubicek-Sutherland, *Mechanisms and consequences of bacterial resistance to antimicrobial peptides.* Drug Resistance Updates, 2016. **26**: p. 43-57.

49. Nawrocki, L.K., K.E. Crispell, and M.S. McBride, *Antimicrobial Peptide Resistance Mechanisms of Gram-Positive Bacteria.* Antibiotics, 2014. **3**(4).

50. Costa, F., et al., *Clinical Application of AMPs*, in *Antimicrobial Peptides: Basics for Clinical Application*, K. Matsuzaki, Editor. 2019, Springer Singapore: Singapore. p. 281-298.

51. Torcato, I.M., et al., *Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2013. **1828**(3): p. 944-955.

52. Han, H.M., R. Gopal, and Y. Park, *Design and membrane-disruption mechanism of charge-enriched AMPs exhibiting cell selectivity, high-salt resistance, and anti-biofilm properties.* Amino Acids, 2016. **48**(2): p. 505-522.

53. Tripathi, A.K., et al., *Identification of GXXXXG motif in Chrysophsin-1 and its implication in the design of analogs with cell-selective antimicrobial and anti-endotoxin activities.* Scientific reports, 2017. **7**(1): p. 3384.

54. Deslouches, B., et al., *Rational design of engineered cationic antimicrobial peptides consisting exclusively of arginine and tryptophan, and their activity against multidrug-resistant pathogens.* Antimicrobial agents and chemotherapy, 2013. **57**(6): p. 2511-2521.

55. Bagheri, M., M. Beyermann, and M. Dathe, *Immobilization reduces the activity of surface-bound cationic antimicrobial peptides with no influence upon the activity spectrum.* Antimicrobial agents and chemotherapy, 2009. **53**(3): p. 1132-1141.

56. Yu, K., et al., *Anti-adhesive antimicrobial peptide coating prevents catheter associated infection in a mouse urinary infection model.* Biomaterials, 2017. **116**: p. 69-81.

57. Gao, G., et al., *Antibacterial surfaces based on polymer brushes: investigation on the influence of brush properties on antimicrobial peptide immobilization and antimicrobial activity.* Biomacromolecules, 2011. **12**(10): p. 3715-3727.

58. Hancock, R.E. and H.-G. Sahl, *Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies.* Nature biotechnology, 2006. **24**(12): p. 1551.

59. Ginsburg, I. and E. Koren, *Are cationic antimicrobial peptides also 'double-edged swords'?* Expert review of anti-infective therapy, 2008. **6**(4): p. 453-462.

60. Matsuzaki, K., *Control of cell selectivity of antimicrobial peptides.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2009. **1788**(8): p. 1687-1692.

61. Pasupuleti, M., et al., *End-tagging of ultra-short antimicrobial peptides by W/F stretches to facilitate bacterial killing.* PLoS One, 2009. **4**(4): p. e5285.

62. Hawrani, A., et al., *Origin of low mammalian cell toxicity in a class of highly active antimicrobial amphipathic helical peptides.* Journal of Biological Chemistry, 2008. **283**(27): p. 18636-18645.

63. Deslouches, B., et al., *De novo generation of cationic antimicrobial peptides: influence of length and tryptophan substitution on antimicrobial activity.* Antimicrobial Agents and Chemotherapy, 2005. **49**(1): p. 316-322.

64. Henriques, S.T., M.N. Melo, and M.A. Castanho, *Cell-penetrating peptides and antimicrobial peptides: how different are they?* Biochemical Journal, 2006. **399**(1): p. 1-7.

65. Yang, S.-T., et al., *Selective cytotoxicity following Arg-to-Lys substitution in tritrpticin adopting a unique amphipathic turn structure.* FEBS letters, 2003. **540**(1-3): p. 229-233.

66. Hicks, R.P., et al., *De novo design of selective antibiotic peptides by incorporation of unnatural amino acids.* Journal of medicinal chemistry, 2007. **50**(13): p. 3026-3036.

67. Zhang, G., et al., *Modification of antimicrobial peptide with low molar mass poly (ethylene glycol).* Journal of biochemistry, 2008. **144**(6): p. 781-788.

68. Imura, Y., M. Nishida, and K. Matsuzaki, *Action mechanism of PEGylated magainin 2 analogue peptide.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2007. **1768**(10): p. 2578-2585.

69. Costa, F., et al., *Covalent immobilization of antimicrobial peptides (AMPs) onto biomaterial surfaces.* Acta biomaterialia, 2011. **7**(4): p. 1431-1440.

70. Eckert, R., et al., *Stability and Activity in Sputum of G10KHc, a Potent Anti-Pseudomonas Antimicrobial Peptide.* Chemical biology & drug design, 2007. **70**(5): p. 456-460.

71. Li, Y., *Production of human antimicrobial peptide LL-37 in Escherichia coli using a thioredoxin–SUMO dual fusion system.* Protein Expression and Purification, 2013. **87**(2): p. 72-78.

72. Parachin, N.S., et al., *Expression systems for heterologous production of antimicrobial peptides.* Peptides, 2012. **38**(2): p. 446-456.

73. Vlieghe, P., et al., *Synthetic therapeutic peptides: science and market.* Drug Discovery Today, 2010. **15**(1): p. 40-56.

74. Kumar, P., J.N. Kizhakkedathu, and S.K. Straus, *Antimicrobial Peptides: Diversity, Mechanism of Action and Strategies to Improve the Activity and Biocompatibility In Vivo.* Biomolecules, 2018. **8**(1): p. 4.

75. Shukla, A., et al., *Controlling the release of peptide antimicrobial agents from surfaces.* Biomaterials, 2010. **31**(8): p. 2348-2357.

76. Bysell, H., et al., *Effect of hydrophobicity on the interaction between antimicrobial peptides and poly (acrylic acid) microgels.* The Journal of Physical Chemistry B, 2010. **114**(3): p. 1307-1313.

77. Wimley, W.C. and K. Hristova, *Antimicrobial Peptides: Successes, Challenges and Unanswered Questions.* The Journal of Membrane Biology, 2011. **239**(1): p. 27-34.

78. Lozeau, L.D., et al., *Concentration-Dependent, Membrane-Selective Activity of Human LL37 Peptides Modified with Collagen Binding Domain Sequences.* Biomacromolecules, 2018.

79. Cloutier, M., D. Mantovani, and F. Rosei, *Antibacterial Coatings: Challenges, Perspectives, and Opportunities.* Trends in Biotechnology, 2015. **33**(11): p. 637-652.

80. Maher, S. and S. McClean, *Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro.* Biochemical pharmacology, 2006. **71**(9): p. 1289-1298.

81. Appendini, P. and J. Hotchkiss, *Surface modification of poly (styrene) by the attachment of an antimicrobial peptide.* Journal of applied polymer science, 2001. **81**(3): p. 609-616.

82. Alexander, T.E., L.D. Lozeau, and T.A. Camesano, *QCM-D Characterization of Time-Dependence of Bacterial Adhesion.* The Cell Surface, 2019: p. 100024.

83. Haynie, S.L., G.A. Crum, and B.A. Doele, *Antimicrobial activities of amphiphilic peptides covalently bonded to a water-insoluble resin.* Antimicrobial Agents and Chemotherapy, 1995. **39**(2): p. 301-307.

84. Li, Y., et al., *Effects of peptide immobilization sites on the structure and activity of surface-tethered antimicrobial peptides.* The Journal of Physical Chemistry C, 2015. **119**(13): p. 7146-7155.

85. Gabriel, M., et al., *Preparation of LL-37-grafted titanium surfaces with bactericidal activity.* Bioconjugate chemistry, 2006. **17**(2): p. 548-550.

86. Glinel, K., et al., *Antibacterial surfaces developed from bio-inspired approaches.* Acta Biomaterialia, 2012. **8**(5): p. 1670-1684.

87. Kügler, R., O. Bouloussa, and F. Rondelez, *Evidence of a charge-density threshold for optimum efficiency of biocidal cationic surfaces.* Microbiology, 2005. **151**(5): p. 1341-1348.

88. Lienkamp, K., et al., *Antimicrobial Polymers Prepared by Ring-Opening Metathesis Polymerization: Manipulating Antimicrobial Properties by Organic Counterion and Charge Density Variation.* Chemistry–A European Journal, 2009. **15**(43): p. 11715-11722.

89. Porcelli, F., et al., *NMR structure of the cathelicidin-derived human antimicrobial peptide LL-37 in dodecylphosphocholine micelles.* Biochemistry, 2008. **47**(20): p. 5565-5572.

90. Wang, K.F., et al., *Characterization of supported lipid bilayer disruption by chrysophsin-3 using QCM-D.* The Journal of Physical Chemistry B, 2011. **115**(51): p. 15228-15235.

91. Wang, K.F., R. Nagarajan, and T.A. Camesano, *Differentiating antimicrobial peptides interacting with lipid bilayer: molecular signatures derived from quartz crystal microbalance with dissipation monitoring.* Biophysical chemistry, 2015. **196**: p. 53-67.

92. Mechler, A., et al., *Specific and selective peptide-membrane interactions revealed using quartz crystal microbalance.* Biophysical Journal, 2007. **93**(11): p. 3907-3916.

93. Uzarski, J.R., et al., *The effects of solution structure on the surface conformation and orientation of a cysteine-terminated antimicrobial peptide cecropin P1.* Colloids and Surfaces B: Biointerfaces, 2008. **67**(2): p. 157-165.

94. Ivanov, I.E., et al., *Creating Antibacterial Surfaces with the Peptide Chrysophsin-1.* ACS Applied Materials & Interfaces, 2012. **4**(11): p. 5891-5897.

95. Iijima, N., et al., *Purification and characterization of three isoforms of chrysophsin, a novel antimicrobial peptide in the gills of the red sea bream, Chrysophrys major.* European Journal of Biochemistry, 2003. **270**(4): p. 675-686.

96. Malmsten, M., K. Emoto, and J.M. Van Alstine, *Effect of Chain Density on Inhibition of Protein Adsorption by Poly(ethylene glycol) Based Coatings.* Journal of Colloid and Interface Science, 1998. **202**(2): p. 507-517.

97. Emoto, K., J.M. Harris, and J.M. Van Alstine, *Grafting poly (ethylene glycol) epoxide to amino-derivatized quartz: effect of temperature and pH on grafting density.* Analytical chemistry, 1996. **68**(21): p. 3751-3757.

98. Sperstad, S.V., et al., *Antimicrobial peptides from marine invertebrates: Challenges and perspectives in marine antimicrobial peptide discovery.* Biotechnology Advances, 2011. **29**(5): p. 519-530.

99. Gao, G., et al., *Biomembrane Interactions Reveal the Mechanism of Action of Surface-Immobilized Host Defense IDR-1010 Peptide.* Chemistry & Biology, 2012. **19**(2): p. 199-209.

100. Hedegaard, S.F., et al., *Fluorophore labeling of a cell-penetrating peptide significantly alters the mode and degree of biomembrane interaction.* Scientific reports, 2018. **8**(1): p. 6327.

101. Fosgerau, K. and T. Hoffmann, *Peptide therapeutics: current status and future directions.* Drug discovery today, 2015. **20**(1): p. 122-128.

102. Batoni, G., et al., *Use of antimicrobial peptides against microbial biofilms: advantages and limits.* Current medicinal chemistry, 2011. **18**(2): p. 256-279.

103. Brogden, N.K. and K.A. Brogden, *Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals?* International journal of antimicrobial agents, 2011. **38**(3): p. 217-225.

104. Hardy, G.J., R. Nayak, and S. Zauscher, *Model cell membranes: Techniques to form complex biomimetic supported lipid bilayers via vesicle fusion.* Current opinion in colloid & interface science, 2013. **18**(5): p. 448-458.

105. Kamaloo, E., *Supported lipid bilayer interactions with nanoparticles, peptides and polymers.* 2018.

106. Kaufmann, S., et al., *Supported lipopolysaccharide bilayers.* Langmuir, 2012. **28**(33): p. 12199-12208.

107. Simpkin, V.L., et al., *Incentivising innovation in antibiotic drug discovery and development: progress, challenges and next steps.* The Journal of antibiotics, 2017. **70**(12): p. 1087-1096.

108. Kimberly Sciarretta, A.O., Adrianus Van-Hengel, John-Arne Rottingen, Joseph Larsen, *Economic Incentives for Antimicrobial Therapy Development: Summary from the Transatlantic Task Force on Antimicrobial Resistance*. 2016: Centers for Disease Control.

109. DiMasi, J.A., H.G. Grabowski, and R.W. Hansen, *Innovation in the pharmaceutical industry: New estimates of R&D costs.* Journal of Health Economics, 2016. **47**: p. 20-33.

110. Ledford, H., *Universities struggle to make patents pay.* Nature News, 2013. **501**(7468): p. 471.

111. Baycan, T. and R.R. Stough, *Bridging knowledge to commercialization: the good, the bad, and the challenging.* The Annals of Regional Science, 2013. **50**(2): p. 367-405.

112. Huang-Saad, A., J. Fay, and L. Sheridan, *Closing the divide: accelerating technology commercialization by catalyzing the university entrepreneurial ecosystem with I-Corps™.* The Journal of Technology Transfer, 2017. **42**(6): p. 1466-1486.

113. Alexander, T.E., Lozeau, Linsday D., Camesano, Terri A., Hoy, Frank, *Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program.* Portland International Center for Management of Engineering and Technology (PICMET), 2019.

114. Blank, S., *Why the lean start-up changes everything.* Harvard business review, 2013. **91**(5): p. 63-72.

115. Osterwalder, A. and Y. Pigneur, *Business model generation: a handbook for visionaries, game changers, and challengers*. 2010: John Wiley & Sons.

116. Blank, S. and B. Dorf, *The startup owner's manual: The step-by-step guide for building a great company*. 2012: BookBaby.

117. York, J., *Putting Lean Startup into Perspective: A Novel Approach for Discovering and Developing a Successful Business Model.* Arch Bus Adm Manag: ABAM-104. DOI, 2018. **10**.

118. Millette, E., *Steve Blank.* 2014.

119. *National Science Foundation - Where Discoveries Begin.* [cited 2019; Available from: https://www.nsf.gov/news/special_reports/i-corps/.

120. Brolin, S., *Global Regulatory Requirements for Medical Devices*. 2008, Akademin för hållbar samhälls-och teknikutveckling.

121. Kaushik, D., et al., *Regulatory perspectives on medical device approval in global jurisdictions.* Journal of Generic Medicines, 2013. **10**(3-4): p. 159-171.

122. Ikeme, A.C., *Nigeria's clinical trial scene.* Applied Clinical Trials, 2008. **17**(6): p. 70.

123. De Maria, C., et al., *Safe innovation: On medical device legislation in Europe and Africa.* Health Policy and Technology, 2018. **7**(2): p. 156-165.

124. Woodyard, A.H., et al., *THE PAPER BLOCK PROJECT: IMPROVING THE DESIGN AND IDENTIFYING MARKET OPPORTUNITIES FOR A SUSTAINABLE COOKING FUEL IN KATUTURA, NAMIBIA.* 2014.

125. Domingue, E.C., et al., *SUSTAINABLE PAPER INSULATION FOR KAMBASHUS IN INFORMAL SETTLEMENTS OF NAMIBIA.* 2012.

126. Dana, L.-P., et al., *Success factors and challenges of grassroots innovations: Learning from failure*. 2019.

127. Kirigia, J.M. and S.P. Barry, *Health challenges in Africa and the way forward.* International archives of medicine, 2008. **1**(1): p. 27-27.

128. Enyinda, C.I. and D. Tolliver, *Taking Counterfeits out of the Pharmaceutical Supply Chain in Nigeria: Leveraging Multilayer Mitigation Approach.* Journal of African Business, 2009. **10**(2): p. 218-234.

129. Tadesse, B.T., et al., *Antimicrobial resistance in Africa: a systematic review.* BMC infectious diseases, 2017. **17**(1): p. 616-616.

130. Scott, R., *II. The Direct Medical Costs of Healthcare-Associated Infections in US Hospitals and the Benefits of Prevention. Centers for Disease Control and Prevention, March 2009*. 2010.

Chapter 3 QCM-D Characterization of Time-Dependence of Bacterial Adhesion

Abstract

Quartz crystal microbalance with dissipation monitoring (QCM-D) is becoming an increasingly popular technique that can be employed as part of experimental and modeling investigations of bacterial adhesion. The usefulness of QCM-D derives from this technique's ability to probe binding and interactions under dynamic conditions, in real time. Bacterial adhesion is an important first step in the formation of biofilms, the control of which is relevant to industries that include shipping, water purification, packaging, and biomedical devices. However, many questions remain unanswered in the bacterial adhesion process, despite extensive research in this area. With QCM-D, multiple variables affecting bacterial adhesion can be studied, including the roles of substrate composition, chemical modification, solution ionic strength, environmental temperature, shear conditions, and time. Recent studies demonstrate the utility of QCM-D in developing new bacterial adhesion models and studying different stages of biofilm formation. We provide a review of how QCM-D has been used to study bacterial adhesion at stages ranging from the first step of bacterial adhesion to mature biofilms, and how QCM-D studies are being used to promote the development of solutions to biofilm formation.

Keywords: QCM-D, biofilm, bacterial adhesion, modeling

3.1 Introduction

Bacterial adhesion is an important step in biofilm formation, which needs to be further understood in order to develop novel solutions to promote or prevent biofilm formation [2, 3]. Undesired biofilm formation causes billions of dollars in economic damage annually in the US and affects a wide variety of sectors, including but not limited to shipping, water treatment (e.g. water supply piping, membranes for water purification, irrigation systems), and healthcare. In the healthcare space alone, infections cause over $45 billion dollars of direct economic damage a year, with a total cost of up to $94 billion [4, 5]. In a 2015 Homeland Security report, the cost of marine biofouling was estimated to be $120 billion dollars annually in the US [6]. A better understanding of the biofilm formation process is needed to help mitigate its economic effects and provide better outcomes for patients, industrial applications, and homeland security.

Bacterial adhesion to a surface is an extremely complex process that begins with: 1) extracellular and intracellular signaling to either recognize a surface or recruit other bacteria to the surface [7, 8], 2) reversible adhesion to the surface via surface proteins such as fibrils and lipopolysaccharide (LPS) [9, 10], 3) irreversible adhesion to the surface, 4) Extracellular matrix (ECM) production and growth of the biofilm changes in protein synthesis and morphological changes [7, 9, 10] and 5) maturation of the biofilm and dispersion [2, 5, 11-15].

The initial step of bacterial adhesion is affected by a number of factors, including external factors like surface energy and topography, and strain specific factors, such as fimbriae and other surface proteins [5, 10, 15]. Not all biofilms are undesired, and in certain applications, such as wastewater treatment and fluidized bed bioreactors, the immobilization of bacteria on surfaces is important for the success of the industrial application [2, 3]. Top fed biofilm reactors have been successfully scaled in order to produce acetic acid for example [16]. More studies are needed before we can rationally design surfaces to either prevent or promote bacterial adhesion. These studies often have limitations due to the techniques used to perform them; therefore,

new techniques and methods such as the Quartz Crystal Microbalance with dissipation monitoring (QCM-D) can be used to better understand the bacterial adhesion process.

3.1.1 Techniques to Measure and Characterize Bacterial Adhesion

Some of the primary techniques that have been used to study bacterial adhesion include atomic force microscopy (AFM), scanning electron microscopy (SEM), flow cells, colony counting, and microcopy [2]. There are many other techniques utilized to study bacterial adhesion, but only a few of the major techniques will be discussed in this review. A more comprehensive list of techniques can be found in Tandogan *et al.* [2] and in Meireles *et al.* [3].

AFM has been very useful in advancing the study of bacterial adhesion as this technique helps provide direct information about adhesive forces and their strength. [8, 17-20]. The AFM tip can be functionalized in order to explore the adhesive forces of different functional groups and coatings. For example, AFM was used to determine the effect of LPS length on bacterial adhesion strength [18]. In another study, *S. epidermidis* was coupled directly to the AFM tip, and adhesion forces between the bacteria and self-assembled monolayers (SAMs) was characterized [21]. This study showed that *S. epidermidis* adhesion was reduced on the SAM surfaces when fibronectin was introduced as a foulant adhesive forces between the bacteria and the fouled SAM surfaces increased. Some limitations of AFM are that the AFM tip needs to be revalidated before each test to ensure there is no contamination, and the technique is low throughput [21]. However, there are recent improvements in methodology in this area. For example, Formosa-Dague et al. have suggested methods to immobilize an array of living bacteria, which is an improvement over earlier AFM protocols involving bacteria. In addition, these authors discuss how to improve the throughput and statistical relevance of AFM measurements [22].

Transmission electron microscopy (TEM) and SEM can offer valuable insight into bacterial adhesion processes, especially in combination with other techniques. In most forms of SEM and TEM, which require an ultrahigh vacuum, samples cannot be reused and the cells need to undergo extensive preparation protocols

[3]. Environmental scanning electron microscopy (ESEM) overcomes some of the challenges of the traditional SEM by allowing for the imaging of a wet sample without the need for a conductive coating [2, 23]. The drawback of ESEM is that the resolution is lower than SEM and often the sample still needs to be coated with a conductive material [2, 23]. Light based microscopy techniques are attractive because they are simple, fast and inexpensive [3, 24, 25]. However, their use is limited to transparent surfaces [3].

One reason to look for another methodology to complement the work being done in AFM and other types of microscopy is that time is an important factor in the bacterial adhesion and biofilm formation processes, but this variable can be difficult to incorporate into static microscopy techniques. Biofilm maturation is dynamic and heavily affected by environmental conditions such as shear forces [19, 26].

QCM-D is able to overcome these shortcomings as it is flow cell technique, which allows for real-time observation of the biofilm formation process [8, 27]. Variables that are difficult to control or change with other techniques, such as flowrate, temperature, ionic strength and nutrient concentration, are easily varied via the QCM-D itself or through the solutions used, offering additional advantages for studying biofilms [8, 27].

3.1.2 Strategies to Prevent Bacterial Adhesion and Biofilm Formation

Many strategies (Figure 1) have been developed to prevent the initial step in biofilm formation, including anti-fouling coatings, contact-active surfaces, increased surface roughness, surface patterning, and biocides [28-30].

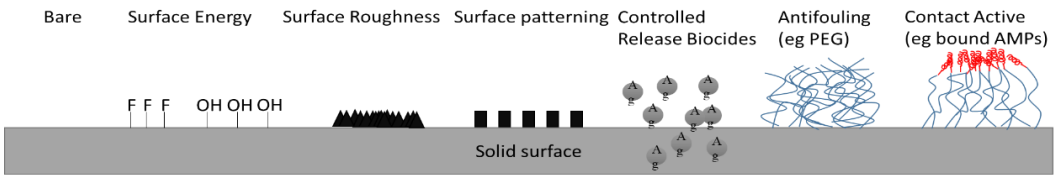


Figure 1. Examples of antibacterial and anti-biofilm surface solutions [28-30]. These include chemical or physical modifications to alter surface energy, surface roughness, surface patterning, controlled release of

biocides (such as silver), antifouling polymers, and contact-active surfaces (bound antimicrobial peptides (AMPs).

The above strategies can be combined for greater and longer term effects, such as the combination of an antifouling polymer with an antimicrobial compound [28]. Types of chemical modifications to a surface include antiadhesive coatings, such as zwitterionic polymers, polyethylene glycol (PEG), hydrogels, and even superhydrophobic coatings, such as slippery liquid infused porous surfaces (SLIPS) [28-30]. These strategies work based on changing the surface energy to discourage protein and bacterial adhesion [31, 32]. In the case of PEG-based coatings, it is theorized that there may be a thermodynamic reason for protein and bacterial repellence due to the preference of PEG to complex with water molecules [31, 32]. This strategy can be effective; however, a perfect coverage is needed to prevent fouling and long-term stability, which is a large manufacturing issue [31, 33, 34]. Contact-active surfaces, which kill bacteria upon interaction, such as quaternary ammonia salts or antimicrobial peptides (AMPs) have shown efficacy [28, 29, 35, 36]. With contact-active surfaces, the challenge of the formation of a conditioning layer by dead bacteria, rendering the surface infective, has not been sufficiently addressed [28, 35, 36]. One strategy to counteract this effect is to combine antifouling polymers with other methods, such as hydrolysis of top layers [37]. This can be overcome by coupling with antifouling polymers or via other mechanisms such as hydrolysis of top layers [37]. Cheng *et al.* [37] used a 'switchable' polymer that has a cationic mechanism of action, but then due to hydrolysis of betaine esters between the quaternary amine and the carboxyl, the polymer transitions from a highly cationic charged molecule to a zwitterionic polymer, which causes the release of the dead bacteria [37].

Other chemical-based surface modification strategies involve the release of biocides, such as silver, but this is limited by the reservoir and diffusion profile of the active ingredient [28, 36, 38-40]. Controlled release has to be used carefully for systems that use traditional antibiotics, as the release itself may promote bacterial resistance [28, 39]. In addition, some of the antibacterial compounds in use (such as copper in the shipping industry) have negative environmental impacts [6, 41].

Introduction of surface roughness and patterning can be used to control bacterial adhesion [42-44]. This strategy is commonly found in nature and can be applied to various materials, including polymers and metals [45]. Feature height and shape have a significant effect on bacterial adhesion; for example, the wing of the clanger cicada has nanoscale pillars (200 nm in height and 60 nm in diameter) that kill bacteria [42-44]. Sharklet™ is one example of a patterned surface based on the topography of shark skin to prevent bacterial adhesion, however, long term efficacy and manufacturability are still being optimized [33, 34]. Due to the diversity of anti-biofouling strategies, there needs to be a way to evaluate how they function in the short- and long-term, as well as to test their application in real-world conditions.

3.2 Factors affecting bacterial adhesion and biofilm maturation evaluated using QCM-D

3.2.1 QCM-D Technique and Sensitivity

QCM-D is a non-destructive flow technique that uses an oscillating piezoelectric quartz crystal sensor to measures changes in frequency (Δf) and dissipation (ΔD) in real time [8, 29, 46]. Recent advances focus on the use of QCM-D to provide more detailed information on bacterial adhesion and interactions with surfaces, under dynamic flow conditions. Variables affecting bacterial adhesion, including the roles of substrate composition, chemical modification, solution ionic strength, environmental temperature, and time can be studied using QCM-D. The QCM-D is a very sensitive technique with the ability to detect mass changes as small as 0.5 ng [18]. Additionally, the viscoelasticity of the adhering protein, chemical film or bacteria can be determined due to the ability of the QCM-D to detect and record energetic losses in the form of dissipation. Overtones are frequencies of the QCM-D sensor that are higher than the fundamental frequency, 5 MHz for AT cut silicone dioxide based sensors, that allow for the interpretation of different phenomena at different energies and penetration depths [47]. Multiple overtones can be measured at once, typically the 3rd through the 11th overtone, with higher overtones measuring processes that are happening closer to the sensor surface (Figure 2). Changes in frequency (Δf) are related to changes in mass (Δm) and changes in dissipation are

related to the rigidity of the film on the surface. This technique allows for real-time analysis, which therefore allows for the study of processes that happen quickly.

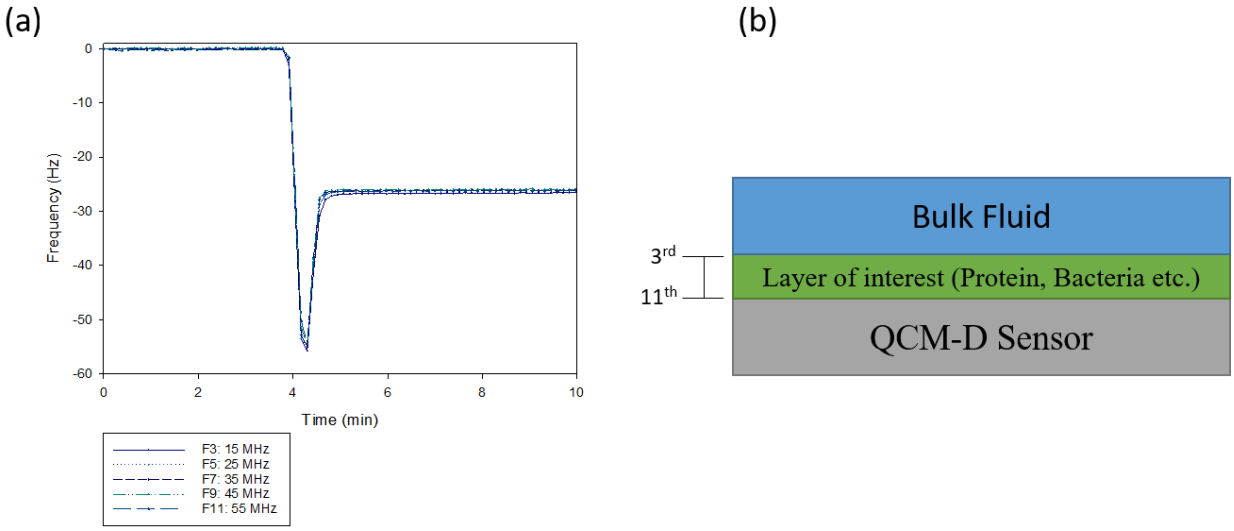


Figure 2. (a) Representative change in frequency for a lipid bilayer adhering to the sensor surface, similar overtone measurement mean similar characteristic structure across the bilayer. The y axis is frequency in hertz and the x axis is time in minutes. Five different overtones are plotted 3^{rd}-11^{th}. (b) Representative image of the penetration depth of various overtones. Higher overtones are more reflective of processes happing near the sensor surface, while lower overtones are representative of processes further from the sensor surface [1].

Numerous studies demonstrate the power of QCM-D in developing new bacterial adhesion models and studying different stages of biofilm formation. Of particular interest is the ability of the QCM-D to identify time-dependent changes in bacterial attachment and morphological changes [8, 11, 12, 27, 48-59]. The sensitivity of the QCM-D allows for the detection of changes that happen in seconds or minutes [25], but QCM-D can also be used for very long time scales, even up to days or weeks [26, 60, 61].

3.2.2 Studies of bacterial adhesion factors using QCM-D

QCM-D experiments can be performed under flow or no-flow conditions, and even a combination of the two [51]. In addition, due to the non-destructive nature of the QCM-D, experiments can be combined with other techniques, including destructive ones, to gather more information than would not be possible if destructive techniques such as SEM were used alone [11, 18, 54, 55, 58, 62]. Leino *et al.* [58] used the QCM-D in combination with both AFM and field emission SEM (FESEM) in order to study the adsorption of *Pseudoxanthomonas taiwanesis* onto cellulose and hemicellulose in the presence of polycations

(poly(diallyldimethyl) ammonium chloride (pDADMAC) and polyacrylamide (C-PAM). They found that the pDADMAC adsorbed as a rigid layer and the C-PAM adhered as a thick loose layer; however, the QCM-D signal did not proportionally respond to the amount of bacteria that was adhered. Then, Leino *et al.* [58] used the AFM and FESEM to directly image the QCM-D sensor which showed that the bacterial cells clusters in "rafts" with large areas with no bacteria in order to supplement their finding on the QCM-D. Since QCM-D is not destructive, there is possibility for its [58] combination with other techniques, such as fluorescent microscopy, AFM and SEM [18, 29].

An example of a strong combination is to combine QCM-D with fluorescence microscopy. In this case, it is possible to calculate the individual contribution that a cell has on the surface, frequency and dissipation shifts, and thus the strength of adhesion for a single cell (Figure 3) [55, 59]. Marcus *et al.* [55] used the window module of the QCM-D, combined with fluorescence microscopy, to study the effect of growth phase on the adhesion of *P. aeruginosa* onto hydrophobic and hydrophilic surfaces at the individual cell level. They found that hydrophobic bacteria formed strong elastic bonds with a hydrophobic surface, and that highest dissipation per cell was found with hydrophilic cells on a hydrophilic surface due to a thin liquid gap between the cell and the surface, thus demonstrating the importance of growth phase on adhesion [55]

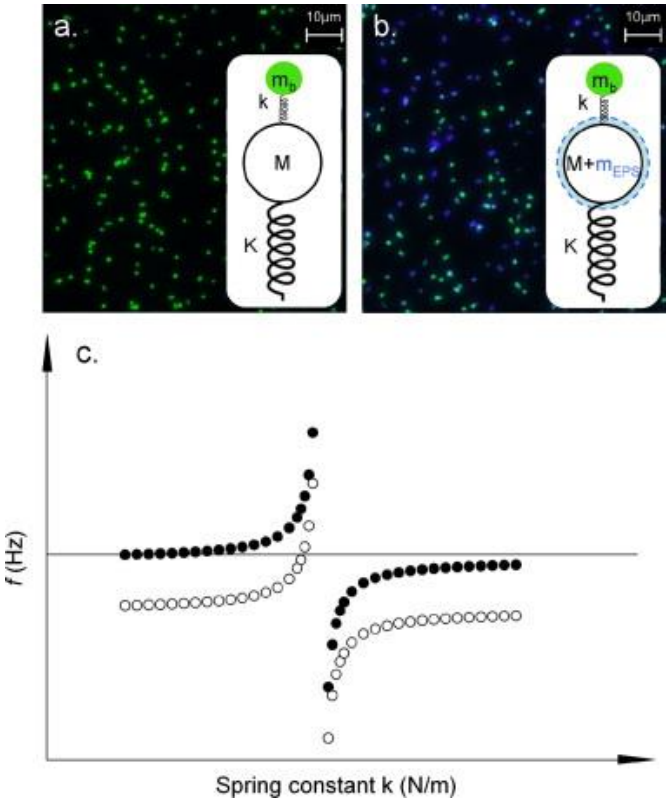


Figure 3. *Reprinted from* Journal of Colloid and Interface Science, 357, Olsson, A. L. J., van der Mei, H. C., Busscher, H. J., Sharma, P. K., Acoustic sensing of the bacterium–substratum interface using QCM-D and the influence of extracellular polymeric substances.,135-138., 2011, with permission from Elsevier. (a) Non-EPS producing strain,(b) EPS producing strain, (c) Maxwell and Voigt-Kelvin Modeling

Understanding the fundamental process by which the bacterium goes from being reversibly attached to irreversibly attached is important for the design of new strategies to prevent bacterial adhesion [2, 5, 7, 10, 46, 49, 58]. QCM-D is useful for studying the bacterial surface proteins that play a role in the transition from a reversible to irreversible adhesion process [18, 24, 25, 27] . For example, Olsson *et al.* [24] used *Streptococcus salivarius* mutants that had surface appendages of known length in order to study the effect that fimbriae length had on adhesion over time. "Bald" bacteria demonstrated a frequency decrease, however, more fibrillation led to the adherence of more bacteria which is in agreement with other studies [12, 17, 24, 62]. It was also found that dissipation was linear when the number of cells was normalized but higher for those with longer fibrils. In a 2010 study, Olsson *et al.* [25], using the same strain of bacteria, demonstrated that the adhesion went from reversible to irreversible in 55 seconds.

The QCM-D can also be used for the study of longer term processes in biofilm formation, including the stage of EPS deposition. For example, Olsson *et al.* [59] measured the EPS secretion of *S. epidermidis* over time, in the absence of growth media. EPS is a major component of biofilms that helps protect the bacteria from antibiotics and other environmental hazards. This study was one of the first to point out how the QCM-D signal is affected by EPS, and this work was built upon with further models to take into account the role of bacterial surface molecules [13, 24, 50, 54, 59]. In addition, the ability to probe viscoelastic properties provides more information on biofilm formation. Reipa et al. studied the long term growth of *P. aeruginosa* in tap water and found that even though thickness of the biofilms did not change, their viscoelastic properties changed as the biofilms matured, becoming less dense and more viscoelastic [61]. They also found that when nutrients were reduced, the biofilms became more rigid [61]. Another study used QCM-D to examine how DNAse 1 and EPS-degrading enzymes break up early stage and mature biofilms [10]. Combining the different types of information that are provided by QCM-D helps in the development of strategies for biofilm removal, which are known to depend on the stage of biofilm formation [6, 10, 30, 63, 64].

Molino *et al.* [65] used the QCM-D to study the interaction of secreted mucilage of two marine diatoms in order to study the first steps in marine biofouling. Using *Craspedostauros australis* (a weak biofouling diatom) and *Amphora coffeaeformis* (a strong and common biofouling diatom), the authors measured the time-dependent deposition of EPS for both species. By calculating the ratio of the change in frequency to the change in dissipation ($\Delta f/\Delta D$) for each diatom, they obtained reproducible signatures for each species that were related to their biofouling properties, and used this to suggest antifouling strategies [65].

Another interesting application of QCM-D was its use to help determine signaling pathways during bacterial adhesion [66]. Using both modified and unmodified *E. coli* strains, Otto *et al.* [66] demonstrated the importance of the Cpx-signaling pathway on adhesion. They determined that NIpE, an outer membrane lipoprotein, plays a major role in bacterial adhesion since when it is absent the Cpx-signaling pathway was not triggered. Both the NIpE protein and the Cpx-pathway are needed for bacterial adhesion and when either were compromised bacteria did not adhere well to any of the surfaces tested [67]. This was apparent in the frequency and dissipation measurements and in the slope of $\Delta D/\Delta f$, where the viscoelastic properties of the wild type cell were significantly different than the mutants [66]. This can lead to the development of solutions to prevent bacterial adhesion by targeting either the protein or the Cpx-pathway. These studies demonstrate the power of the QCM-D to systematically help determine the signaling pathways via bacterial mutants and the effect of the outside environment, such as hydrophobicity, on bacterial adhesion [11, 24, 67]. This could lead to novel design of anti-biofilm molecules that could be developed in a traditional pharmaceutical setting.

3.3. QCM-D Evaluation of Modified Surfaces that Affect Bacterial Adhesion

With a better understanding of how bacteria adhere to surfaces, there is great interest in using QCM-D, among other techniques, to study and design surfaces that prevent adhesion [2, 7, 10, 14, 30, 35]. Some strategies are based on physical changes to the structure of the surface, for example, patterning (Sharklet™), roughness, and mechanical deformation [33, 34]. Other methods involve changing the surface energy via modification of the surface through plasma etching or chemically binding polymers such as polyethylene

glycol or fluoropolymers [28-30]. Surfaces can also be modified to allow for direct killing of bacteria through the release of a biocide or a coating of a biocide such as antimicrobial peptides or quaternary ammonia salts [28, 35, 36, 38]. Contreras *et al.* [49] used the QCM-D to study the ability of sodium dodecyl sulfate (SDS) to clean various functionalized surfaces and found that there was mixed success in its use at removing conditioning proteins and polysaccharides, BSA and alginate, from the surfaces tested. The ease at which surfaces can be cleaned is also a factor in the design of anti-fouling surfaces.

3.3.1 Passive strategies to prevent bacterial adhesion

Surface patterning is one strategy to prevent or promote bacterial adhesion, and a number of QCM-D studies have examined the effect of patterning on factors that affect bacterial adhesion [16, 68, 69]. For example, Welle *et al.* [68] used the QCM-D to study protein adhesion onto patterned polystyrene surfaces. By application of the Voigt-Kelvin viscoelastic model, they determined the amount of protein adsorption and the viscoelastic properties of the film. Cerf *et al.* [69] examined the nano-mechanical properties of live and dead bacteria on nano-patterned surfaces using QCM-D and AFM. QCM-D was useful for measuring the electrostatic binding forces and determining which surfaces the bacteria adhered best to. In another study, Thickett *et al.* [70] examined the effect of collagen adsorption onto micro-patterned surfaces of two different polymers, polystyrene and poly(*N*-vinylpyrrolidone). From the frequency shift, it was determined that collagen adsorption was much higher on the polystyrene surface, adsorbed viscoelastically (increase in dissipation) on both surfaces, and could be removed via rinsing, as observed by a return in frequency close to the baseline value [70].

For antifouling surfaces such as PEG, zwitterionic polymers, and SAMs, the QCM-D can be useful for characterization deposition and hydration of the film itself, in addition to studying how bacteria interact with the surface. The surface characterizations can be used to directly incorporate variables, including polymer density, hydration, and thickness, to models of bacterial adhesion. For example, Yandi *et al.* [71] showed that they could use the QCM-D to study film thickness and hydration level and couple that directly with the effect

on bacterial adhesion. The thickness of random poly(HEMA-co-PEG10MA) copolymer brushes affected antifouling behavior directly, and film hydration was a critical component. Reduced antifouling performance was caused by a lower hydration capacity of thin films, and that entanglement and crowding of thicker films reduced hydration capacity [71]. Many QCM-D based studies of antifouling substrates focus on protein adhesion as that is often considered to be a primary step before bacterial adhesion [19, 49, 68, 72-74]. Eichler *et al.* [57] studied the effectiveness of a dendrimer-based coating to repel bacteria after being conditioned with salivary proteins. They were able to determine the mass of protein that adhered to each film using an extended Voigt model. When the mass of the water adsorption was taken into account, the dendrimer polymers outperformed the other coatings and reduced bacterial adhesion. QCM-D was essential to uncovering this result because this technique allowed the authors to measure both the mass of adsorbed protein and the mass of the adsorbed water layer. As another example, Muszanska *et al.* [75] studied a family of triblock copolymers, consisting of a hydrophobic core and hydrophilic end groups. They determined the hydrated thickness and viscoelastic properties of the polymers and demonstrated how the polymers were conformationally adhered to the surface, which each play a role in bacterial adhesion.

3.3.2 Active strategies to prevent bacterial adhesion

Contact active surfaces are an attractive method to prevent biofilm formation, often these strategies employ compounds that are active in solution which make studying their bound properties and mechanisms of action important [29, 76]. A growing area of research is contact active surfaces based on antimicrobial peptides (AMPs), due to their broad spectrum activity and low likelihood of bacterial resistance [29]. Lozeau *et al.* [29] demonstrated the ability of the QCM-D to determine the mechanism of action of a covalently bound antimicrobial peptide chrysophsin-1, and showed how peptide activity was related to the length of the tether molecule. By combining QCM-D with fluorescence microscopy, the authors showed that for the short tether, the key variable affecting activity was the cationic charge of the antimicrobial peptide, making it interact with the membrane. However, for a sufficiently long tether, a pore forming mechanism controlled the interaction

between the antimicrobial peptide and the membrane, and this was similar to the behavior of C-CHY1 in solution [29]. Atefyekta *et al.* [76] used QCM-D to quantify the mass of tethered peptide on a surface, as well as to study the stability of the bond. Similarly, Yoshinari *et al.* [77] used QCM-D with a window module in order to quantify AMP adsorption on PMMA and directly couple that to antimicrobial activity. Etienne *et al.* [78] used the QCM-D to study the effect the number of AMP-impregnated polyelectrolyte layers had on the killing of *Micrococcus luteus* and *Escherichia coli*, and determined that 10 layers was optimal compared to 5 or fewer layers.

3.4 Methods and Modelling of QCM-D Systems: Current Status and Future Directions

The QCM-D is a non-destructive flow technique that uses an oscillating piezoelectric quartz crystal sensor to measures changes in frequency (Δf) and dissipation (ΔD) in real time [79-81]. The changes in frequencies can be related directly to changes in mass through the Sauerbrey equation for rigid films and adhesion. However, these models often fall short when trying to directly measure bacterial adhesion due to the complex processes that are involved in bacterial adhesion, as well as coupled resonance [8, 12]. Coupled resonance is most commonly observed when there is a positive shift in frequency is observed unexpectedly when mass is in fact being added to the system. Fimbriae and proteins on the bacterial surface can cause this phenomenon [8]. Further, if the inertial and spring elastic force of a bacteria on the surface is perfectly balanced, then Δf will be zero [8]. The Voigt-Kelvin extended viscoelastic model corrects the Sauerbrey estimations for higher energy dissipation by adding terms to the Δf relation to mass and ΔD relation to film rigidity [8, 12]. Analysis of frequency and dissipation changes at different overtones, corresponding to differing penetration depths (250 nm maximum), allows for the study of the effect of fimbriae length or other bacterial surface molecules on adhesion [24, 25, 59], which provides for real-time mechanistic study of the bacterial adhesion process [8].

3. 4.1 Models based on change in frequency

Many of the models that improve on the Sauerbrey relationships make use of data from multiple overtones, which makes the model output more accurate and informative [8, 29, 56, 62, 71]. For example, the Johannsmann model improves on the Sauerbrey model for biopolymers, by incorporating multiple overtones, and uses a statistical regression line of each overtone related to mass change in order to determine the overall changes in mass [56, 81]. New models should include the ability to incorporate different overtones in order to fully use all the information that the QCM-D acquires, temperature, frequency and dissipation separation at different overtones [8, 56]. Continued model development is needed to better interpret complex systems studied using the QCM-D [8].

Fundamental changes in frequencies can be related directly to changes in mass through the Sauerbrey equation (1) for rigid films and adhesion [18, 20, 29, 79]. Where f_o is the resonant frequency of the sensor, A is the piezoelectrically-active area of the crystal, ρ_q is the density of quartz and μ_q shear modulus of quartz for AT-cut crystals.

(1) $$\Delta f = -\frac{2f_o^2}{A\sqrt{\rho_q \mu_q}}\Delta m$$

This can be simplified, where C is a constant of 17.8 ng/cm^2/Hz, where the resonant frequency is 5 MHz, and n is the corresponding overtone number.

(2) $$\Delta m = -\frac{C \cdot \Delta f}{n}$$

The QCM-D also measures the energetic loss, or energy dissipation of the crystal, which is directly related to the viscoelasticity of deposited films. Dissipation (D) can be described by the following equation (3).

(3) $$D = \frac{G^{"}}{2\pi G'}$$

Where G' and $G^{"}$ represent the loss and storage modulus respectively. The Sauerbrey relation is only valid for rigid films where the dissipation is close to zero. For non-rigid films the Sauerbrey relation will often

underestimate adhered mass and cannot be used for films with significant dissipation. Bacterial cells are covered with various proteins, sugars and other functional groups which can allow for non-rigid contact/ adhesion with the QCM-D sensor surface. This also causes coupled resonance which can present a positive frequency shift even though there is an addition of mass to the surface.

The Johnnsmann model differs from the Sauerbrey model in that it accounts for viscoelasticity of the adsorbed layer [56, 82]. The model can be mathematically represented as:

(4) $$\delta\hat{f} \approx -f_o * \frac{1}{\pi\sqrt{\rho_q u_q}}(2\pi f \rho d + \hat{J}(f) * \frac{(2\pi f)^3 \rho_f^2 d^3}{3})$$

Where $\delta\hat{f}$ is the shift in complex frequency, d is the thickness of the film, f is the is the resonance frequency of the crystal and $\hat{J}(f)$ is the complex shear compliance. The equivalent mass can be calculated using equation 5.

(5) $$\hat{m}^* = m^o + \hat{J}(f)\frac{\rho_q(2\pi f)^2 d^2}{3}$$

Where $\hat{m}^*$ is the equivalent mass and m^o is the true sensed mass.

3.4.2 Models based on change in frequency and energetic losses (dissipation)

The Voigt-Kelvin extended viscoelastic model corrects the Sauerbrey estimations for higher energy dissipation by adding terms to the Δf relation to mass and ΔD relation to film rigidity[12, 29]. The Voigt-Kelvin-Model cam be represented as a purely elastic spring and a purely viscous dampener in parallel as seen in Figure 4 and represented mathematically below in equation 6.

(6) $$\Delta f + \frac{i\Delta D f_o}{2} = \frac{f_F m_p}{\pi Z_q} \cdot N_p \left[\frac{\omega_s^3(\omega_p^2-\gamma^2)-\omega_s\omega_p^4}{(\omega_s^2-\omega_p^2)^2+\omega_s^2\omega_\gamma^2} + i\frac{\omega_s^4\gamma}{(\omega_s^2-\omega_p^2)^2+\omega_s^2\omega_\gamma^2}\right]$$

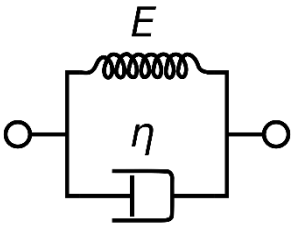


Figure 4. A Voigt-Kelvin material can be represented by a purely elastic spring connected in parallel with a purely vicious dampener. By Pekaje at English Wikipedia (Transferred from en.wikipedia to Commons.) [Public domain], via Wikimedia Commons

Where f_o is the resonant frequency of the sensor, f_F is the fundamental frequency of the crystal, m_p is the inertial mass of the particle or bacteria, ω_p is the resonance angular frequency for the particle or bacteria, ω_s is the sensor angular frequency, Z_q is the acoustic impedance of an AT-cut quartz crystal and N_p is the number of adhering particles or bacteria. Where $\gamma = \frac{\xi}{m_p}$ and ξ is the drag coefficient.

Similarly the Maxwell model can be used as an improvement to the Sauerbrey model when dissipation is high [12]. The Maxwell model can be represented as a purely elastic spring and a purely viscous dampener in series as seen in Figure 5 and represented mathematically below in equation 7 [12].

(7) $$\Delta f + \frac{i\Delta D f_o}{2} = \frac{f_F N_p}{\pi Z_q} \cdot \left[i\omega_s m_p \frac{1}{1 - \frac{\omega_s^2}{\omega_p^2} + \frac{i\omega}{\gamma}} \right]$$

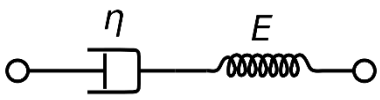


Figure 5. Maxwell representative model. A Maxwell material can be represented by a purely elastic spring connected in series with a purely vicious dampener. By Pekaje at English Wikipedia (Transferred from en.wikipedia to Commons.) [Public domain], via Wikimedia Commons

It is not clear if the Voigt-Kelvin or Maxwell models are better for studying bacteria with the QCM-D [12]. van der Westen *et al.* [12] found that including polydispersity in the models had no effect for "bald" *S. salivarius* HBC12. For the *S. salivarius* HB7 the Maxwell equation led to a mass four times than what was calculated with the Voigt model. While these models can be useful and the output parameters of frequency

and dissipation can be used to study bacterial adhesion and biofilm maturation as seen in the studies that have been presented. It is clear that better models are needed in order to fully harness the power of the QCM-D. Several groups have attempted to do so [83, 84].

3.4.3 Models based on ΔD vs. Δf $\Delta D / \Delta f$

Many studies are able to use the ratio of $\Delta D / \Delta f$ in order to gain insight into the bacterial adhesion and biofilm maturation process [8, 27, 50, 52, 85]. $\Delta D / \Delta f$ can be used to represent the viscoelastic properties of the deposited bacteria or biofilm [8, 27, 50, 52, 85]. For example, Tarnapolsky *et al.* [8] used $\Delta D / \Delta f$ to determine the structure of the biofilm as well as use the $\Delta D / \Delta f$ profile to determine two different biofilm formation regimes. By combining this analysis with other QCM-D outputs this ratio can be used to detect very sensitive processes including conformational changes, for antifouling surfaces, biofilm removal strategies, and restructuring of the bacterial cells or biofilm itself [8, 13, 29, 56, 71, 75].

However, these models often fall short when trying to directly measure bacterial adhesion due to the complex processes that are involved in bacterial adhesion as well as coupled resonance [8, 12]. Due to the various fimbriae and proteins on the bacterial surface coupled resonance may cause a positive shift in frequency which is not what would be expected due to an addition of mass on the sensor surface. This is the major challenge when modeling bacteria with the QCM-D; however, valuable information about adhesion strength and progression, based on the dissipation parameter, can be gleaned and has allowed for new model development.

4.4 Recent models: dissipative elastic contributions and frequency with overtone dependence

Tarnapolsky *et al.* [8] sought to develop a model for QCM-D by describing the interaction of a freely oscillating sphere in an unbounded fluid. They then tested and validated their model by first using abiotic spheres under various surface chemistries and ionic strengths then validated it by using *P. fluorescens.* A graphical representation of the model can be seen in Figure 6.

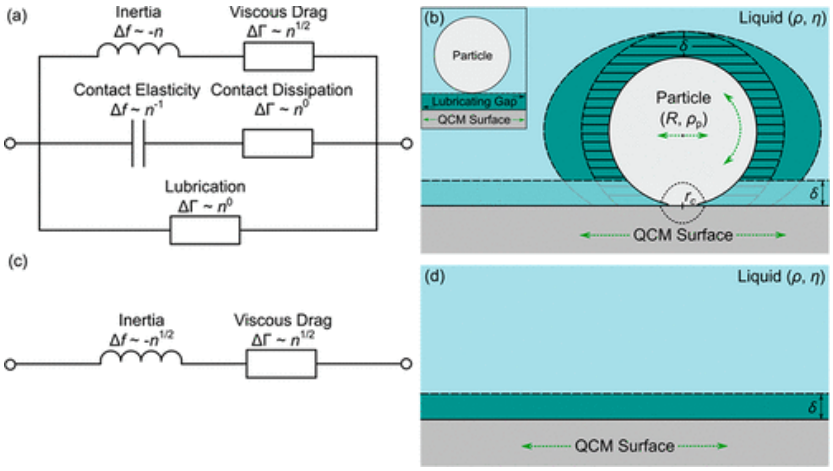


Figure 6. "Reprinted (adapted) with permission from Tarnapolsky, A. and Freger, V. (2018). Modeling QCM-D Response to Deposition and Attachment of Microparticles and Living Cells. Analytical Chemistry. Copyright 2018 American Chemical Society." The equivalent electrical circuit for the model developed by Tarnapolsky and Freger with (a) and (b) representing a particle or bacteria adhearing to a crystal and (c) and (d) representing a bare crystal.

Mathematically their model is as follows:

(8) $$Z_L^* = \frac{\sigma}{\dot{u}}$$

Where $Z_L^*$is the complex load impedance when a particle contacts the QCM-D sensor surface and σ is the tangential stress and $\dot{u}$ is the tangential velocity at the sensor–solution interface [8]. Frequency of a given overtone is related to Z_L^* by the following equation:

(9) $$\Delta f_n = -\frac{f_F}{\pi Z_q} Im(\Delta Z_L^*), \Delta\Gamma_n = \frac{f_F \eta}{2} \Delta D_n = \frac{f_F}{\pi Z_q} Re(\Delta Z_L^*)$$

Where Δf_n is the frequency, $\Delta\Gamma_n$ is the bandwidth and ΔD_n is the dissipation shift shifts for overtone n, and f_F = 5 MHz is the fundamental frequency, and Z_q = 8.8 × 10^6 kg m^{-2} s^{-1} is the acoustic impedance of the AT-cut quartz sensor [8]. Further derivation and simplification can be found in Tarnapolsky et al. [8].

Tarnapolsky *et al.* [8] were able to successfully develop a model that incorporates both dissipative and elastic contributions and frequency with overtone dependence. More importantly this allows for the direct

study of dissipation due to contact of the cell with the surface, eliminating dissipation due to bulk bacterial motion under flow. This allows for better analysis of both bacterial adhesion and biofilm analysis in future studies. While this new model is promising further validation under more conditions, different surfaces, temperature and bacterial strains must be tested. Additional models will likely be necessary for conditions under different use techniques such as those for testing antifouling and antimicrobial surfaces.

3.5 Conclusions/Outlooks

The QCM-D is a powerful technique that allows for the study of bacterial interactions with surfaces under many types of conditions. Given the cost of unwanted biofilms in various industries and rising antibiotic resistance, it is clear that there is a need to better understand the biofilm formation process in order to develop strategies for their control. A number of variables that include temperature, pH, ionic strength, and surface chemistry are easily changed and manipulated using the QCM-D, either through experimental design or via pre and post treatments of the sensor surface. Additionally, since this technique is nondestructive, it can be combined readily with other techniques for more powerful and in depth analysis. QCM-D can give information at scales that range from the single cell level to the biofilm level. The most important advantages are the range of properties that can be measured (viscoelasticity, conformational changes), and the real-time analysis with a time resolution of less than 1s.

Data analysis is critical to interpretation of the phenomena happening on the surface of the sensor of the QCM-D, thus it is important to use the proper model for each experimental situation. The Sauerbrey equation can be used for studies that examine the adhesion of various conditioning proteins onto rigid surfaces where the proteins of interest do not adsorb significant amount of water (are not viscoelastic). Factors such as ionic strength, pH, temperature and hydrophobicity can be studied. The Voigt-Kelvin viscoelastic and Maxwell models allow for the study of more viscoelastic and complex systems and are therefore, appropriate to use when studying antimicrobial and anti-adhesive surfaces. Studies of well-defined bacteria, such as those

with known fibril lengths, can also be studied using the Voigt-Kelvin viscoelastic and Maxwell models, with Maxwell modelling more appropriate for more fluid-like viscoelastic materials [86]. Additionally, $\Delta f/\Delta D$ and $\Delta \mathrm{D}/\Delta f$ combined with careful overtone analysis can yield deep insights into the properties of bacterial adhesion, biofilm maturity, distance from the surface and even signature profiles [8, 50, 85]. The QCM-D alone can yield powerful insights but it can be used in combination with other techniques due to its non-destructive nature to yield even more power results. New models such as the one developed by Tarnapolsky *et al.* [8] allow for more powerful insight and can allow for more standalone QCM-D studies however it still needs more validation. In the meantime other models should continue to be developed and ideally a general model that is sufficiently complete but simple can be developed and adopted. With further model development the QCM-D has the ability to be a major and important tool in the development of the next generation of industrial, medical and biomaterials.

This work was published in **Alexander, T.E.**, L.D. Lozeau, and T.A. Camesano, QCM-D Characterization of Time-Dependence of Bacterial Adhesion. The Cell Surface, 2019: p. 100024.

References

1. Mechler, A., et al., *Specific and selective peptide-membrane interactions revealed using quartz crystal microbalance.* Biophysical Journal, 2007. **93**(11): p. 3907-3916.
2. Tandogan, N., et al., *Characterization of Bacterial Adhesion and Biofilm Formation*, in *Antimicrobial Coatings and Modifications on Medical Devices*, Z. Zhang and V.E. Wagner, Editors. 2017, Springer International Publishing: Cham. p. 67-95.
3. Meireles, A., et al., *Methods to study microbial adhesion on abiotic surfaces.* AIMS Bioengineering, 2015. **2**(4): p. 297-309.
4. Scott, R.D., *The direct medical costs of healthcare-associated infections in US hospitals and the benefits of prevention.* 2009.
5. Römling, U., et al., *Microbial biofilm formation: a need to act.* Journal of internal medicine, 2014. **276**(2): p. 98-110.
6. McClay, T., et al., *Vessel biofouling prevention and management options report*. 2015, COAST GUARD NEW LONDON CT RESEARCH AND DEVELOPMENT CENTER.
7. Khelissa, S., et al., *Bacterial contamination and biofilm formation on abiotic surfaces and strategies to overcome their persistence.* J Mater Environ Sci, 2017. **8**: p. 3326-3346.
8. Tarnapolsky, A. and V. Freger, *Modeling QCM-D Response to Deposition and Attachment of Microparticles and Living Cells.* Analytical Chemistry, 2018.
9. Goulter, R., I. Gentle, and G. Dykes, *Issues in determining factors influencing bacterial attachment: a review using the attachment of Escherichia coli to abiotic surfaces as an example.* Letters in applied microbiology, 2009. **49**(1): p. 1-7.
10. Kostakioti, M., M. Hadjifrangiskou, and S.J. Hultgren, *Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era.* Cold Spring Harbor perspectives in medicine, 2013. **3**(4): p. a010306-a010306.

11. Gutman, J., et al., *Bacterial Attachment and Viscoelasticity: Physicochemical and Motility Effects Analyzed Using Quartz Crystal Microbalance with Dissipation (QCM-D).* Environmental Science & Technology, 2013. **47**(1): p. 398-404.

12. van der Westen, R., et al., *Elastic and viscous bond components in the adhesion of colloidal particles and fibrillated streptococci to QCM-D crystal surfaces with different hydrophobicities using Kelvin–Voigt and Maxwell models.* Physical Chemistry Chemical Physics, 2017. **19**(37): p. 25391-25400.

13. Kwon, K.D., et al., *Model Bacterial Extracellular Polysaccharide Adsorption onto Silica and Alumina: Quartz Crystal Microbalance with Dissipation Monitoring of Dextran Adsorption.* Environmental Science & Technology, 2006. **40**(24): p. 7739-7744.

14. Flemming, H.-C., et al., *Biofilms: an emergent form of bacterial life.* Nature Reviews Microbiology, 2016. **14**: p. 563.

15. Tuson, H.H. and D.B. Weibel, *Bacteria–surface interactions.* Soft matter, 2013. **9**(17): p. 4368-4380.

16. Qureshi, N., et al., *Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates.* Microbial cell factories, 2005. **4**(1): p. 24.

17. Strauss, J., et al., *Binding, inactivation, and adhesion forces between antimicrobial peptide cecropin P1 and pathogenic E. coli.* Colloids and Surfaces B: Biointerfaces, 2010. **75**(1): p. 156-164.

18. Strauss, J., Y. Liu, and T.A. Camesano, *Bacterial adhesion to protein-coated surfaces: An AFM and QCM-D study.* JOM, 2009. **61**(9): p. 71-74.

19. Marcotte, L. and M. Tabrizian, *Sensing surfaces: Challenges in studying the cell adhesion process and the cell adhesion forces on biomaterials.* IRBM, 2008. **29**(2): p. 77-88.

20. Camesano, T.A., Y. Liu, and M. Datta, *Measuring bacterial adhesion at environmental interfaces with single-cell and single-molecule techniques.* Advances in Water Resources, 2007. **30**(6-7): p. 1470-1491.

21. Liu, Y., J. Strauss, and T.A. Camesano, *Adhesion forces between Staphylococcus epidermidis and surfaces bearing self-assembled monolayers in the presence of model proteins.* Biomaterials, 2008. **29**(33): p. 4374-4382.

22. Formosa-Dague, C., et al., *Generation of living cell arrays for atomic force microscopy studies.* nature protocols, 2015. **10**(1): p. 199.

23. Stokes, D.J., *Recent advances in electron imaging, image interpretation and applications: environmental scanning electron microscopy.* Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, 2003. **361**(1813): p. 2771-2787.

24. Olsson, A.L.J., et al., *Influence of Cell Surface Appendages on the Bacterium−Substratum Interface Measured Real-Time Using QCM-D.* Langmuir, 2009. **25**(3): p. 1627-1632.

25. Olsson, A.L.J., et al., *Novel Analysis of Bacterium−Substratum Bond Maturation Measured Using a Quartz Crystal Microbalance.* Langmuir, 2010. **26**(13): p. 11113-11117.

26. Chen, M.-Y., et al., *Towards real-time observation of conditioning film and early biofilm formation under laminar flow conditions using a quartz crystal microbalance.* Biochemical Engineering Journal, 2010. **53**(1): p. 121-130.

27. Otto, K., H. Elwing, and M. Hermansson, *Effect of Ionic Strength on Initial Interactions ofEscherichia coli with Surfaces, Studied On-Line by a Novel Quartz Crystal Microbalance Technique.* Journal of Bacteriology, 1999. **181**(17): p. 5210-5218.

28. Adlhart, C., et al., *Surface modifications for antimicrobial effects in the healthcare setting: a critical overview.* Journal of Hospital Infection, 2018.

29. Lozeau, L.D., T.E. Alexander, and T.A. Camesano, *Proposed mechanisms of tethered antimicrobial peptide chrysophsin-1 as a function of tether length using QCM-D.* The Journal of Physical Chemistry B, 2015. **119**(41): p. 13142-13151.

30. Chen, M., Q. Yu, and H. Sun, *Novel strategies for the prevention and treatment of biofilm related infections.* International journal of molecular sciences, 2013. **14**(9): p. 18488-18501.

31. Banerjee, I., R.C. Pangule, and R.S. Kane, *Antifouling coatings: recent developments in the design of surfaces that prevent fouling by proteins, bacteria, and marine organisms.* Advanced materials, 2011. **23**(6): p. 690-718.

32. Damodaran, V.B. and N.S. Murthy, *Bio-inspired strategies for designing antifouling biomaterials.* Biomaterials research, 2016. **20**(1): p. 18.

33. Chung, K.K., et al., *Impact of engineered surface microtopography on biofilm formation of Staphylococcus aureus.* Biointerphases, 2007. **2**(2): p. 89-94.

34. Graham, M.V. and N.C. Cady, *Nano and microscale topographies for the prevention of bacterial surface fouling.* Coatings, 2014. **4**(1): p. 37-59.

35. Glinel, K., et al., *Antibacterial surfaces developed from bio-inspired approaches.* Acta Biomaterialia, 2012. **8**(5): p. 1670-1684.

36. Gao, G., et al., *Biomembrane Interactions Reveal the Mechanism of Action of Surface-Immobilized Host Defense IDR-1010 Peptide.* Chemistry & Biology, 2012. **19**(2): p. 199-209.

37. Cheng, G., et al., *A switchable biocompatible polymer surface with self-sterilizing and nonfouling capabilities.* Angewandte Chemie, 2008. **120**(46): p. 8963-8966.

38. Tiller, J.C., *Antimicrobial surfaces*, in *Bioactive surfaces*. 2010, Springer. p. 193-217.

39. Pickard, R., et al., *Antimicrobial catheters for reduction of symptomatic urinary tract infection in adults requiring short-term catheterisation in hospital: a multicentre randomised controlled trial.* The lancet, 2012. **380**(9857): p. 1927-1935.

40. Bonfill, X., et al., *Efficacy and safety of urinary catheters with silver alloy coating in patients with spinal cord injury: a multicentric pragmatic randomized controlled trial. The ESCALE trial.* The Spine Journal, 2017. **17**(11): p. 1650-1657.

41. O'Neill, J., *Antimicrobial resistance: tackling a crisis for the health and wealth of nations.* Rev. Antimicrob. Resist, 2014. **20**: p. 1-16.

42. Song, F., H. Koo, and D. Ren, *Effects of Material Properties on Bacterial Adhesion and Biofilm Formation.* Journal of Dental Research, 2015. **94**(8): p. 1027-1034.

43. Vasudevan, R., et al., *Microscale patterned surfaces reduce bacterial fouling-microscopic and theoretical analysis.* Colloids and Surfaces B: Biointerfaces, 2014. **117**: p. 225-232.

44. Perera-Costa, D., et al., *Studying the Influence of Surface Topography on Bacterial Adhesion using Spatially Organized Microtopographic Surface Patterns.* Langmuir, 2014. **30**(16): p. 4633-4641.

45. Scardino, A.J. and R. de Nys, *Mini review: Biomimetic models and bioinspired surfaces for fouling control.* Biofouling, 2011. **27**(1): p. 73-86.

46. Busscher, H.J., et al., *Interfacial re-arrangement in initial microbial adhesion to surfaces.* Current Opinion in Colloid & Interface Science, 2010. **15**(6): p. 510-517.

47. Goka, S., et al., *Multimode quartz crystal microbalance.* Japanese Journal of Applied Physics, 2000. **39**(5S): p. 3073.

48. Speight, R.E. and M.A. Cooper, *A Survey of the 2010 Quartz Crystal Microbalance Literature.* Journal of Molecular Recognition, 2012. **25**(9): p. 451-473.

49. Contreras, A.E., et al., *Studying the role of common membrane surface functionalities on adsorption and cleaning of organic foulants using QCM-D.* Environmental science & technology, 2011. **45**(15): p. 6309-6315.

50. Sweity, A., et al., *Relation between EPS adherence, viscoelastic properties, and MBR operation: Biofouling study with QCM-D.* Water Research, 2011. **45**(19): p. 6430-6440.

51. Schofield, A.L., et al., *Real-time monitoring of the development and stability of biofilms of Streptococcus mutans using the quartz crystal microbalance with dissipation monitoring.* Biosensors and Bioelectronics, 2007. **23**(3): p. 407-413.

52. Zhou, T., et al., *The Quartz Crystal Microbalance as a Continuous Monitoring Tool for the Study of Endothelial Cell Surface Attachment and Growth.* Biotechnology Progress, 2000. **16**(2): p. 268-277.

53. Poitras, C. and N. Tufenkji, *A QCM-D-based biosensor for E. coli O157:H7 highlighting the relevance of the dissipation slope as a transduction signal.* Biosensors and Bioelectronics, 2009. **24**(7): p. 2137-2142.

54. Olsson, A.L.J., M.R. Mitzel, and N. Tufenkji, *QCM-D for non-destructive real-time assessment of Pseudomonas aeruginosa biofilm attachment to the substratum during biofilm growth.* Colloids and Surfaces B: Biointerfaces, 2015. **136**: p. 928-934.

55. Marcus, I.M., et al., *Pseudomonas aeruginosa Attachment on QCM-D Sensors: The Role of Cell and Surface Hydrophobicities.* Langmuir, 2012. **28**(15): p. 6396-6402.

56. Feldötö, Z., T. Pettersson, and A. Dėdinaitė, *Mucin− electrolyte interactions at the solid− liquid interface probed by QCM-D.* Langmuir, 2008. **24**(7): p. 3348-3357.

57. Eichler, M., et al., *The impact of dendrimer-grafted modifications to model silicon surfaces on protein adsorption and bacterial adhesion.* Biomaterials, 2011. **32**(35): p. 9168-9179.

58. Leino, T., et al., *Adsorption of bacteria and polycations on model surfaces of cellulose, hemicellulose and wood extractives studied by QCM-D.* Colloids and Surfaces B: Biointerfaces, 2011. **86**(1): p. 131-139.

59. Olsson, A.L.J., et al., *Acoustic sensing of the bacterium–substratum interface using QCM-D and the influence of extracellular polymeric substances.* Journal of Colloid and Interface Science, 2011. **357**(1): p. 135-138.

60. Nivens, D.E., et al., *Long-term, on-line monitoring of microbial biofilms using a quartz crystal microbalance.* Analytical Chemistry, 1993. **65**(1): p. 65-69.

61. Reipa, V., J. Almeida, and K.D. Cole, *Long-term monitoring of biofilm growth and disinfection using a quartz crystal microbalance and reflectance measurements.* Journal of Microbiological Methods, 2006. **66**(3): p. 449-459.

62. Olofsson, A.-C., M. Hermansson, and H. Elwing, *Use of a Quartz Crystal Microbalance To Investigate the Antiadhesive Potential of N-Acetyl-l-Cysteine.* Applied and Environmental Microbiology, 2005. **71**(5): p. 2705-2712.

63. Simoes, M., L.C. Simões, and M.J. Vieira, *A review of current and emergent biofilm control strategies.* LWT-Food Science and Technology, 2010. **43**(4): p. 573-583.

64. Gall, I., M. Herzberg, and Y. Oren, *The effect of electric fields on bacterial attachment to conductive surfaces.* Soft Matter, 2013. **9**(8): p. 2443-2452.

65. Molino, P.J., et al., *Utilizing QCM-D to characterize the adhesive mucilage secreted by two marine diatom species in-situ and in real-time.* Biomacromolecules, 2006. **7**(11): p. 3276-3282.

66. Otto, K. and T.J. Silhavy, *Surface sensing and adhesion of Escherichia coli controlled by the Cpx-signaling pathway.* Proceedings of the National Academy of Sciences, 2002. **99**(4): p. 2287-2292.

67. Otto, K. and M. Hermansson, *Inactivation of ompX causes increased interactions of type 1 fimbriated Escherichia coli with abiotic surfaces.* Journal of bacteriology, 2004. **186**(1): p. 226-234.

68. Welle, A., et al., *Photo-chemically patterned polymer surfaces for controlled PC-12 adhesion and neurite guidance.* Journal of Neuroscience Methods, 2005. **142**(2): p. 243-250.

69. Cerf, A., et al., *Nanomechanical properties of dead or alive single-patterned bacteria.* Langmuir, 2009. **25**(10): p. 5731-5736.

70. Thickett, S.C., et al., *Micropatterned substrates made by polymer bilayer dewetting and collagen nanoscale assembly support endothelial cell adhesion.* Soft Matter, 2012. **8**(39): p. 9996-10007.

71. Yandi, W., et al., *Hydration and Chain Entanglement Determines the Optimum Thickness of Poly(HEMA-co-PEG10MA) Brushes for Effective Resistance to Settlement and Adhesion of Marine Fouling Organisms.* ACS Applied Materials & Interfaces, 2014. **6**(14): p. 11448-11458.

72. Guo, S., et al., *Tailoring Polyelectrolyte Architecture To Promote Cell Growth and Inhibit Bacterial Adhesion.* ACS Applied Materials & Interfaces, 2018. **10**(9): p. 7882-7891.

73. Boulmedais, F., et al., *Polyelectrolyte multilayer films with pegylated polypeptides as a new type of anti-microbial protection for biomaterials.* Biomaterials, 2004. **25**(11): p. 2003-2011.

74. Song, L., et al., *Contribution of Adsorbed Protein Films to Nanoscopic Vibrations Exhibited by Bacteria Adhering through Ligand–Receptor Bonds.* Langmuir, 2015. **31**(38): p. 10443-10450.

75. Muszanska, A.K., et al., *Pluronic–lysozyme conjugates as anti-adhesive and antibacterial bifunctional polymers for surface coating.* Biomaterials, 2011. **32**(26): p. 6333-6341.

76. Atefyekta, S., et al., *Antibiofilm Elastin-like Polypeptide Coatings-Functionality, Stability, and Selectivity.* Acta Biomaterialia, 2018.

77. Yoshinari, M., et al., *Adsorption behavior of antimicrobial peptide histatin 5 on PMMA.* Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2006. **77B**(1): p. 47-54.

78. Etienne, O., et al., *Multilayer polyelectrolyte films functionalized by insertion of defensin: a new approach to protection of implants from bacterial colonization.* Antimicrobial agents and chemotherapy, 2004. **48**(10): p. 3662-3669.

79. Sauerbrey, G., *The use of quarts oscillators for weighing thin layers and for microweighing.* Z. Phys., 1959. **155**: p. 206-222.

80. Steinem, C. and A. Janshoff, *Piezoelectric sensors*. Vol. 5. 2007: Springer Science & Business Media.

81. Johannsmann, D., *Viscoelastic, mechanical, and dielectric measurements on complex samples with the quartz crystal microbalance.* Physical Chemistry Chemical Physics, 2008. **10**(31): p. 4516-4534.

82. Johannsmann, D., *Viscoelastic analysis of organic thin films on quartz resonators.* Macromolecular Chemistry and Physics, 1999. **200**(3): p. 501-516.

83. Olsson, A.L., et al., *Probing colloid–substratum contact stiffness by acoustic sensing in a liquid phase.* Analytical chemistry, 2012. **84**(10): p. 4504-4512.

84. Song, L., et al., *Nanoscopic vibrations of bacteria with different cell-wall properties adhering to surfaces under flow and static conditions.* ACS nano, 2014. **8**(8): p. 8457-8467.

85. Fredriksson, C., et al., *The Piezoelectric Quartz Crystal Mass and Dissipation Sensor: A Means of Studying Cell Adhesion.* Langmuir, 1998. **14**(2): p. 248-251.

86. Voinova, M.V., et al., *Viscoelastic acoustic response of layered polymer films at fluid-solid interfaces: continuum mechanics approach.* Physica Scripta, 1999. **59**(5): p. 391.

Specific Aim 1: Chapter 4 Mechanistic Analysis of Tethered Antimicrobial Peptide Chrysophsin-1 as a Function of Tether Length Using QCM-D

Abstract

Rising antibiotic resistance has led to a call for the development of alternative antibiotics. Antimicrobial peptides (AMPs) are promising, but their potential has not been fully explored because of toxicity and lack of stability *in vivo*. Multiple recent studies have focused on surface immobilization of AMPs to maximize antimicrobial activity and stability while mitigating toxicity. We covalently tethered cysteine-modified chrysophsin-1 (C-CHY1) via PEG of three molecular weights, 866, 2000 and 7500. Quartz crystal microbalance with dissipation (QCM-D) was used to characterize thickness and grafting density of tethered C-CHY1, which were related to its activity against *Staphylococcus aureus* and *Escherichia coli* and found to be important in determining mechanisms leading to activity. The PEG 866 tether promoted an antimicrobial mechanism that caused displacement of positive cations from bacterial membranes. The PEG 7500 tether maintained C-CHY1's ability to effectively form membrane pores, promoting the highest activity. When AMP was tethered with PEG 2000, antimicrobial activity was limited, apparently because neither mechanism of AMP activity was able to occur with this tether. Using QCM-D we were able to calculate thickness and density of PEG-tethered C-CHY1 and correlate it with antimicrobial effectiveness in real-time and determine the mechanisms by which tethered C-CHY1 acts.

4.1 Introduction

Increasing bacterial resistance due to the overuse of conventional antibiotics has led to an alarming rate of nosocomial infection and a desperate call for the development alternative antibiotics. The CDC estimates that these infections kill at least 23,000 people per year [1]. Among the most promising alternatives are antimicrobial peptides (AMPs). AMPs are short peptide sequences (10-50 amino acids) and most are amphipathic and cationic (+2 to +9 charge) [2-5]. They are associated with the innate immunity of several species [2-5]. Continuous exposure to a diverse array of pathogens requires AMPs to have broad spectrum activity against bacteria, fungi, viruses, and protozoa [5, 6]. AMPs are recognized for their potent activity against common gram negative and gram positive microbes, such as *Escherichia coli (E. coli)* and *Staphylococcus aureus* (*S. aureus*), which are common in hospitals and community environments. The development of antimicrobial resistance to current antibiotics has been identified as a serious threat [1].

AMPs use different mechanisms to kill bacteria than traditional antibiotics, making the development of bacterial resistance much less likely; however, these mechanisms differ between peptides and are not fully understood [4, 7-11]. Most proposed mechanisms involve physical AMP-membrane interactions, with AMPs using their positive charge and hydrophobicity to target anionic bacterial membranes, create pores, cause cell leakage, and eventually promote cell [12-15]. In some cases the AMP may be nonspecifically cytotoxic, which poses a hurdle for their clinical implementation [4, 10, 11, 16]. For example, human-derived LL-37 negatively affects keratinocyte viability at 10 μM [17], melittin displays significant toxicity against epithelial cells at only 1.2 μM [18], and FDA-approved nisin demonstrates toxicity to epithelial cells at 89.9 μM [18]. To further understand how to reduce AMP toxicity for their use in the clinic, AMP mechanisms of action must be better understood [18].

Nosocomial infection plays a major role in causing failure of some biomedical devices. Each year in the United States, 5-10% out of 2 million fracture fixation devices and 1-2% of the 600,000 implanted joint prostheses become infected, leading to extended hospital stays and revision surgeries, ultimately costing over $250 million [19-21]. The best way to combat these infections is to prevent bacterial colonization in the first

place [22-24]. Due to their broad spectrum activity and ability to be chemically manipulated, AMPs may provide a solution for a wide variety of applications including preventing the infection of medical devices. Tethering AMPs to medical devices may offer a promising way to prevent infection.

Recent studies have explored several strategies of tethering AMPs onto a variety of surfaces as means to localize and maintain activity while minimizing toxicity; however, the effect of tethering on activity of AMPs is still debated [24-36]. AMP activity is dependent on peptide orientation and secondary structure. This was demonstrated by Gabriel *et al.* [25], who showed that the orientation of tethered LL-37 significantly affected antimicrobial activity and that only LL-37 tethered to a flexible tether via its N-terminus had any significant bactericidal activity [25]. Surface concentration or density of the AMP also may effect antimicrobial activity [37] but activity is not solely a function of AMP density. In some cases, increasing AMP density had no effect on activity [38]. Density can be limited by the number of functional groups available on the surface or due to steric hindrance [39]. Therefore, it is important to determine surface density to further our mechanistic understanding of tethered AMPs [36, 39]. Tether length is also thought to be crucial for maintaining AMP activity while reducing toxicity [26-28]. Some studies suggest that tethering is peptide-dependent, meaning certain AMPs require longer tethers while others do not [26, 27, 39, 40]. However, other studies showed that AMPs tethered to short spacers can still maintain significant activity but via a different mechanism than their counterparts in solution [41-43]. Flexibility of the spacer is another important parameter that may affect the activity of tethered AMPs [25, 26, 34]. For example, Gabriel *et al.* [25] also found that antimicrobial activity could only be achieved with tethered LL-37 via a flexible PEG molecule, where they suggested flexibility was needed to allow for proper orientation of the peptide to the bacterial surface [25].

Studying tethered AMP surface density, tethering behaviors and activity can require the use of multiple, often complex, destructive, and expensive techniques [36, 39]. The different methods used to characterize tethered AMP structure-activity relationships have led to inconsistent results [39]. Many methods are destructive or lack the ability to measure dynamic interaction of peptide surface attachment in real-time,

such as NMR and X-ray crystallography, and do not allow for subsequent antimicrobial testing [39, 44]. It is important to use a technique that is able to measure tethered AMP properties in real-time, particularly surface density and thickness, as they all factor into antimicrobial activity of the system [25].

The quartz-crystal microbalance with dissipation monitoring (QCM-D) has emerged as a convenient non-destructive method to measure surface interactions in real-time between AMPs and membranes [28, 45]. QCM-D measures changes in frequency (Δf) and dissipation (ΔD) that correspond to changes in mass deposition (Δm) and film rigidity, respectively, on the surface of oscillating piezoelectric quartz crystal sensors with nanogram-level sensitivity. It is a non-destructive flow technique, allowing for coupling to other experiments. For example, Ivanov *et al.* [28] used QCM-D to monitor surface attachment and interactions of tethered AMPs, as well as to quantify *ex situ* bacterial viability, allowing for characterization of antimicrobial activity. Chrysophsin-1 (CHY1) tethered to SiO_2 via a biocompatible and flexible poly(ethylene glycol) (PEG) linker demonstrated 82 ± 11% killing of *E. coli* when covalently immobilized via an N-terminus cysteine modification (C-CHY1)[28]. This was compared to greater than 99.9% killing by CHY1 in solution [28]. This study demonstrated that C-CHY1 could be tethered while maintaining good activity.

In the present study, we used QCM-D to characterize the activity of C-CHY1 against gram positive and gram negative bacteria when covalently tethered to silicon dioxide (SiO_2) via three different PEG linker molecule lengths, compared with physically-adsorbed CHY1 and solution-based C-CHY1. Our goal was to use QCM-D to determine the mechanism of tethered C-CHY1 as it relates to tether length and peptide surface density. This understanding will allow for more rational design of tethered AMPs that are broadly antimicrobial and minimally toxic, bringing them closer to preventing nosocomial device-associated infection in the clinic.

4.2 Experimental and/or Theoretical Methods

4.2.1 Bacterial Strains and Culturing. *Escherichia coli HB101* (ATCC 33694) and *Staphylococcus aureus* (ATCC 48366) were cultured overnight in Luria-Bertani broth (20 g/L). For QCM-D and toxicity

studies, cells were harvested at the late logarithmic phase in their growth curve, as confirmed by absorbance measurements (OD_{600} = 0.7-1.0) (Thermo Scientific USA, Waltham, MA USA). The cells were centrifuged at 1284 x g (Centrific Thermo Scientific, Waltham, MA USA) and re-suspended in 0.01 M, pH 7.2 phosphate buffered saline (PBS) (Sigma Aldrich, St. Louis, MO USA) twice, and then diluted 100-fold to approximately 10^8 cfu/mL.

4.2.2 Peptides. Chrysophsin-1 (CHY1; FFGWLIKGAIHAGKAIHGLIHRRRH) and the N-terminus cysteine-modified chrysophsin-1 (C-CHY1; CFFGWLIKGAIHAGKAIHGLIHRRRH) were purchased from Bachem Americas, Inc. (Torrance, CA USA). The peptides were received as a lyophilized trifluoroacetate salt at greater than 85% purity confirmed by high performance liquid chromatography. Solutions of 5 g/L CHY1 and C-CHY1 were made in PBS (pH 7.2) and PBS supplemented with 1 mM ethylenediaminetetraacetic acid (PBS/EDTA; pH 7.2) as a chelating agent respectively, and stored at -20^{o}C. All buffer solutions for storage, dilutions and experimentation were degassed by sonication under vacuum for 30 min prior to their use. The minimum inhibitory concentrations (MIC) [46] of these peptides against several common microbes were found prior to tethering (supplementary data).

4.2.3 QCM-D: Covalent Linking of C-CHY1. SiO_2-coated quartz crystal sensors from Biolin Scientific (Stockholm, Sweden) were used as immobilization platforms for the modified C-CHY1. Before use, SiO_2 sensors were cleaned in the QCM-D at 40^{o}C using ethanol, DI water, 2% sodium dodecyl sulfate (w/v), DI water again, and then nitrogen dried. Lastly, sensor surfaces were treated for 2 min using an oxygen plasma cleaner (SPI Supplies, PA USA) to both clean and functionalize the surface. The SiO_2 crystals were then silanized by submerging in a 10% (v/v) (3-Aminopropyl)-trimethoxysilane (APTMS) in methanol solution for 20 min. Each sensor was then rinsed twice thoroughly with methanol and DI water and placed in each QCM-D chamber.

Changes in frequency (Δf, Hz) and dissipation (ΔD, $x10^{-6}$ Hz) at the 3rd, 5th, 7th, 9th and 11th overtones were monitored at a constant 23°C in all chambers using a Q-Sense E4 QCM-D system (Biolin Scientific,

Stockholm, Sweden). All flow rates for the solutions were at 0.1 mL/min unless otherwise noted, and all volumes given are on a per chamber basis. PBS/EDTA (pH 7.2) buffer was used to establish a stable baseline measurement. Maleimide PEG *N*-hydroxysuccinimide ester molecules (MAL-PEG-NHS) with molecular weights (MW) of 866 (ThermoScientific, Waltham MA, USA), 2000, or 7500 (JenKem Technology USA Inc., Allen, TX USA) were purchased. These will be referred to as PEG 866, PEG 2000 and PEG 7500, respectively. One mL of 100 µM MAL-PEG-NHS was flowed through the QCM-D and subsequently incubated for 30 min. Crystals were then rinsed with 1.2 mL of the PBS/EDTA (pH 7.2) buffer. Similarly, 1.25 mL of 10 µM C-CHY1 solution was then flown over the sensors and allowed to incubate for 30 min. To rinse excess C-CHY1 off the surface and prepare for the introduction of 2 mL bacteria, a 45 min PBS rinse at 0.3 mL/min was first flowed through the QCM-D. The dilute bacterial solution was allowed to incubate for 1 hour, followed by a final 2 mL PBS (pH 7.2) rinse. The crystals were removed from the chambers and placed in individual petri dishes containing 0.85% (w/v) NaCl solution for bacterial viability testing.

4.2.4 QCM-D Control Experiments: Physical Adsorption of CHY1. Similarly, SiO_2-coated sensors were used for physical adsorption of unmodified CHY1 and were prepared as described above. For this experiment there was no APTMS functionalization or PEG flow, thus, after cleaning, the crystals were placed immediately into the QCM-D, PBS (pH 7.2) was used to establish a baseline and 1.25 mL of 10 µM CHY1 solution was introduced. Then, flow was stopped and CHY1 was incubated for 30 min. The rest of the protocol described in the previous section was then followed, including the 0.3 mL/min PBS rinse, bacterial flow, bacterial incubation and final PBS rinse. The crystals were removed from the chambers and placed in individual petri dishes containing 0.85% (w/v) NaCl solution for bacterial viability testing. For the next type of control, crystals that had never been coated with APTMS were cleaned and placed into the QCM-D chambers without functionalization. Starting with a PBS rinse, the crystals were exposed to bacteria solution for 1 hour and then rinsed before imaging. For the APTMS control, crystals were cleaned and functionalized with APTMS. Then the procedure continued starting with a PBS rinse (pH 7.2), bacteria introduction,

incubation, and a final rinse before imaging. For the PEG control, the crystals were cleaned and functionalized with APTMS. A baseline was established, PEG was flown and then incubated. Peptide was not introduced in this type of control experiment. Separate PEG control experiments were performed for each PEG size. All experiments were repeated at least three times.

4.2.5 Bacterial Mortality. Bacterial mortality was determined immediately after the final rinse of the QCM-D experiment. Crystals were stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies Corp, NY USA) in 2 mL of 0.85% (w/v) NaCl solution with 5 μM SYTO 9 and 30 μM propidium iodide for 15 min. The crystals were rinsed once using 0.85% (w/v) NaCl solution to remove any excess dye and then kept in 1 mL saline to keep hydrated for imaging. The crystals were imaged at 20x objective using fluorescein isothiocyanate (526 nm) and Texas Red filters (620 nm) under a Nikon Eclipse E400 fluorescence microscope (Melville, NY USA). A minimum of 5 locations on each crystal were examined for live and dead bacteria, totaling at least 10 images. Images were analyzed using ImageJ Software (http://rsbweb.nih.gov/ij/) to produce a merged image from which the percent mortality of the cells was determined.

4.2.6 QCM-D: Data Modeling. The viscoelasticity of deposited material demonstrated by the QCM-D raw data was used to determine which model to use in estimating parameters of the system such as mass of attachment (ng), layer thicknesses (nm) and peptide areal mass (ng/cm^2). The brush may be thought to be made up of two layers, first is APTMS plus PEG and second is C-CHY1. The former demonstrates near-zero dissipation values, thus, the Sauerbrey equation for rigid surfaces (Eqn. 1) applies, where Δm is inversely related to Δ*f*. For the Sauerbrey model, ΔD and film rigidity are related using Eqn. 2.

$$\Delta f = -\frac{2f_0^2}{A\sqrt{\rho_q \mu_q}}\Delta m \qquad (1)$$

$$\Delta D = \frac{G'}{2\pi G''} \qquad (2)$$

Where f_0 is the fundamental frequency of the quartz crystal, 5 MHz; A is the active crystal surface area; ρ_q is the density of quartz, 2.648 g/cm^3; and μ_q is the shear modulus of the crystal, 2.947x10^{11} g/cm·s^2; G'' is the loss modulus and G' is the storage modulus of the film attached to the crystal surface. Thus, decreases in Δf demonstrate an addition of mass and higher ΔD values indicate a softer film. The Sauerbrey model was applied to time points from the flow of PEG through the flow of C-CHY1. A "maximum" PEG attachment was also calculated using the minimum frequency value between the two time points for comparison (supplemental data).

Once C-CHY1 is grafted, the layer becomes significantly more dissipative as demonstrated by raw QCM-D data, which has been seen in previous work. Thus, parameters of the system were modeled using the Voigt-Kelvin extended viscoelastic model in Q-Tools software (Biolin Scientific, Stockholm, Sweden). This model corrects the Sauerbrey estimations for higher energy dissipation by adding terms to the Δf relation to mass (Eqn. 3) and ΔD relation to film rigidity (Eqn. 4).

$$\Delta f = -\frac{\eta_L}{2\pi\delta_L m_q} - f_o \frac{m_f}{m_q}\left[1 - \frac{2}{\rho_f}\left(\frac{\eta_L}{\delta_L}\right)^2 \frac{G''}{G'^2+G''^2}\right] \qquad (3)$$

$$\Delta D = \frac{\eta_L}{n\pi f_o \delta_L m_q} + \frac{m_f}{m_q}\left[\frac{4}{\rho_f}\left(\frac{\eta_L}{\delta_L}\right)^2 \frac{G'}{G'^2+G''^2}\right] \qquad (4)$$

Where η_L is the viscosity of the bulk liquid assumed to be water (kg/m·s); δ_L and δ_f are the decay lengths of the acoustic wave in the bulk liquid and film (m), respectively; m_q and m_f are the (kg), respectively; ρ_f is the film density (kg/m^3). The layer was modeled to get numerical outputs for layer viscosity, density, and shear modulus (η_m, ρ_m, and μ_m), and film thickness. All overtones were modeled at once. The bulk liquid, predominantly PBS or PBS/EDTA, was assumed to have the same viscosity and density as water at 23°C. Model step size and output ranges were changed based on calculated theoretical values using estimated extended molecule size, and the lowest chi square value (χ^2) was taken. Once values of thickness (nm) and density (kg/m^3) were found with the model, the two were multiplied to calculate areal mass in ng/cm^2. The

Sauerbrey estimate (Eqn. 1 and 2) for the QCM-D crystal surface area for the grafted C-CHY1 layer was used to compare with modeled values (supplemental data).

Because the long grafted layers are thought to be associated with water and the QCM-D modeling does not account for this, reported literature values [47] of associated water molecules with PEG monomers were used to model the mass of the system accounting for water (supplemental data).

4.3 Results

4.3.1 Addition of Mass Suggests Successful Grafting of C-CHY1 to the Surface via PEG Tethers. QCM-D was used to monitor covalent attachment in real-time of the PEG spacer followed by C-CHY1 peptide to the APTMS-functionalized SiO_2 surface (Figure 1). According to the Sauerbrey calculation, the Δm due to PEG spacers is between 2 and 100 ng per crystal (Table 1; Figure 2). The mass of PEG attached decreases with increasing tether length (Figure 2). PEGylated surfaces show a rigid surface (ΔD values from $+1x10^{-6}$ to $+2x10^{-6}$ Hz) compared to the bulk fluid baseline, validating the use of Eqn. 1 and 2 for PEG layer calculations. When C-CHY1 is introduced, there is a rapid decrease of Δf corresponding to a Δm of 0.8 to 2.2 μg (Table 1) bound to the surface. This step is accompanied by a large increase in ΔD from near $+2x10^{-6}$ to $+20x10^{-6}$ Hz after the introduction of C-CHY1. This suggests that C-CHY1 causes a less rigid, more hydrated "brush-like" film compared with PEGylated surfaces. A high ΔD from the addition of C-CHY1 makes the Sauerbrey assumption less accurate (Figure 2). Comparatively, CHY1 introduction leads to a Δf decrease corresponding a Δm of about 0.4 μg of CHY1 being physically-adsorbed on the surface. During the rinse, a slightly higher increase in Δf of +5 Hz suggests some of the CHY1 is removed, consistent with its non-covalent nature, but about 50% of the peptide remains adsorbed. The ΔD does not deviate from the baseline value, suggesting that a rigid surface is maintained throughout the entire CHY1 experiment.

Table 1: Sauerbrey and Voigt-Kelvin Calculations of Mass Attachment, Layer Thickness and Areal Mass for each PEG Tether Length

[a]Type of tether molecule	PEG attachment ng	Chrysophsin-1 attachment ng		PEG/C-CHY1 layer thickness (nm)[c]	C-CHY1 areal mass (ng/cm^2)	
	[b]Sauerbrey	[b]Sauerbrey	[c]Voigt-Kelvin		[b]Sauerbrey	[c]Voigt-Kelvin
No Tether (PHYS-ADSORB)	--	204.9 ± 7.99	373.3 ± 58.6	2.50 ± 0.49	260.9 ± 10.2	475.3 ± 74.6
PEG 866	98.1 ± 7.88	874.0 ± 48.8	835.2 ± 168	6.59 ± 1.14	1113 ± 62.2	1063 ± 215
PEG 2000	41.8 ± 6.77	2238 ± 60.4	2284 ± 113	17.6 ± 1.32	2850 ± 76.9	2909 ± 144
PEG 7500	2.46 ± 4.58	1753 ± 43.9	1870 ± 176	21.9 ± 1.86	2232 ± 55.0	2381 ± 224

[a]All values represent the mean for the 3rd through 11th overtones. And error reported represents standard error for n>3 surfaces total, corrected for sample size by dividing standard deviation by the square root of the number of replicates.

[b]The mass attached for PEG and C-CHY1 molecules is estimated using the assumption of a rigid surface (low ΔD values) for columns labeled "Sauerbrey Estimate" using equations 1 and 2

[c]For C-CHY1, viscoelasticity of the system was corrected for using the Voigt-Kelvin model, equations 3 and 4 to calculate attachment mass (ng), layer thickness (nm), and areal mass (ng/cm^2).

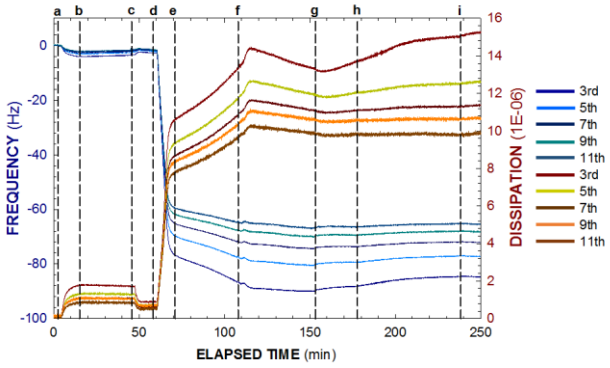


Figure 1: Typical QCM-D Response to Grafting C-CHY1 to SiO_2 Surfaces via a PEG Tether Molecule. Changes in frequency (Hz) in blue are shown on the primary axis, and changes in dissipation ($x10^{-6}$ Hz) in red are shown on the secondary axis versus elapsed time (min) recorded with QCM-D for the 3rd through 11th overtones. After a baseline is established with PBS/EDTA (pH 7.2), a change in the experimental condition is demonstrated at each time stamp (--): (a) flow of PEG tether molecules; (b) stoppage of flow for 30 min; (c) PBS/EDTA (pH 7.2) buffer rinse; (d) C-CHY1 molecules; (e) stoppage of flow again for 30 min; (f) PBS (pH 7.2) rinse at a comparatively higher flow rate for 45 min; (g) bacteria exposure; (h) stoppage of flow for 1 hour allowing peptide-bacteria interactions; (i) final PBS (pH 7.2) rinse prior to *ex situ* cell viability imaging of the QCM-D sensor surface.

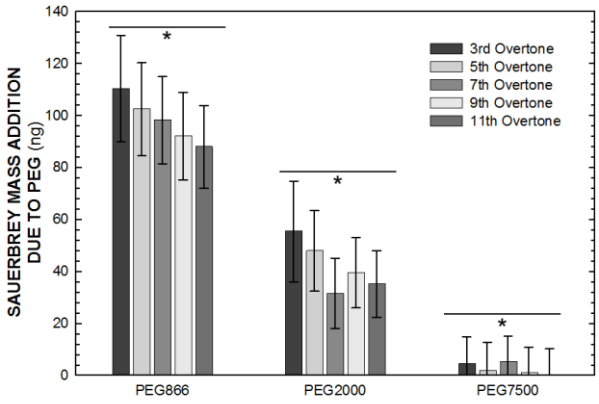


Figure 2: Mass Attachment of PEG versus PEG Size. Mass attachment in ng was estimated for each PEG molecular weight at each overtone using the Sauerbrey equation. Reported error is standard error, corrected for sample size by dividing standard deviation by the square root of the number of replicates, n>3. Two-way ANOVA was performed. Statistical significance (*) was found between all sizes of PEG ($p<0.05$) but not for the individual overtones within each group.

4.3.2 Film Thickness Increases with Tether Length. Thickness of the covalently-bound PEG and C-CHY1 film on the crystal surface was modeled using the Voigt-Kelvin model (Table 1; Figure 3), Eqn. 3 and 4. A

theoretical maximum thickness using the assumption of the full extension of PEG and C-CHY1 was calculated using molecular bond lengths and found to be 8.8 nm [28], 17.8 nm and 58.05 nm for PEG 866, 2000 and 7500, respectively. Similar to what has been seen for the shortest spacer length in prior work from our laboratory [28], we found that tethered C-CHY1 via PEG 866 is approximately 6.59 ± 1.14 nm thick. As expected this thickness increases with increasing PEG size, from 17.6 ± 1.32 nm for PEG 2000 and 21.9 ± 1.86 nm for PEG 7500. These numbers suggest that the PEG was almost fully extended for PEG 866 and 2000 because of their similarity to the calculated values. For PEG 7500, the modeled thickness is 38% of theoretical maximum thickness, suggesting that the PEG is not fully extended. This could be due to PEG 7500 chains interacting with neighboring chains and peptides, entangling with each other [48]. Thickness of the physically-adsorbed CHY1 film was similarly modeled. The thickness is 2.5 ± 0.5 nm, consistent with prior results from our laboratory [28].

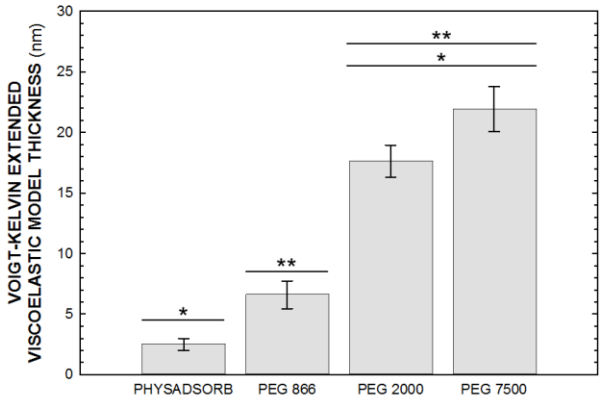


Figure 3: Thickness of PEG and C-CHY1 Layers, Calculated using Voigt-Kelvin Extended Viscoelastic Modeling. The Voigt-Kelvin viscoelastic model was used to calculate the layer thickness. Reported error is standard error, corrected for sample size by dividing standard deviation by the square root of the number of replicates, n>3. One-way ANOVA was performed. Significance was observed between the PHYSADSORB control and PEG 2000 or PEG 7500 (*) using a post-hoc Dunnett Test and was also observed with a post-hoc

Tukey Test ($p<0.05$) between PEG 866 and PEG 2000 or PEG 7500 (**), but not between PEG 2000 and PEG 7500.

4.3.3 PEG Tether Mass Plus Associated Water Molecules Determine the Areal Mass of the Film. For each of the PEG spacer lengths, the areal mass was determined using the density (kg/m^3) obtained by the Voigt-Kelvin viscoelastic model. The areal masses calculated for n=3 replicates were 1063 ± 215 ng/cm^2, 2909 ± 144 ng/cm^2, and 2381 ± 223 ng/cm^2 for PEG 866, 2000 and 7500, respectively (Table 1; Figure 4a). This areal mass includes the mass of the PEG spacer, attached C-CHY1, and the trapped buffer solution. Under the Voigt-Kelvin model, we assume the following: a Newtonian bulk fluid, a laterally homogenous and evenly distributed film, a soft and viscoelastic film (high ΔD), and an adsorbed layer is coupled to the sensor. This model utilizes the ΔD and Δf values contributed by the entire film on the surface, including associated buffer molecules, to determine thickness and density (Eqn. 3 and 4).

For comparison, the Sauerbrey equation was used to calculate the mass addition between PEG flow and bacteria flow to find the overall grafted mass at each overtone. Then, this was divided by the crystal surface area (Figure 4b). This estimation was a good fit for the areal mass of grafted PEG, peptide and associated water. Interestingly, the dense packing of PEG 866 demonstrated in Figure 2 limits its trapped buffer molecules to those associated with PEG monomers only. Thus, we found that using the total system mass and the mass of PEG monomer-associated water molecules[47] was also a good fit for lower tether lengths (supplemental data).

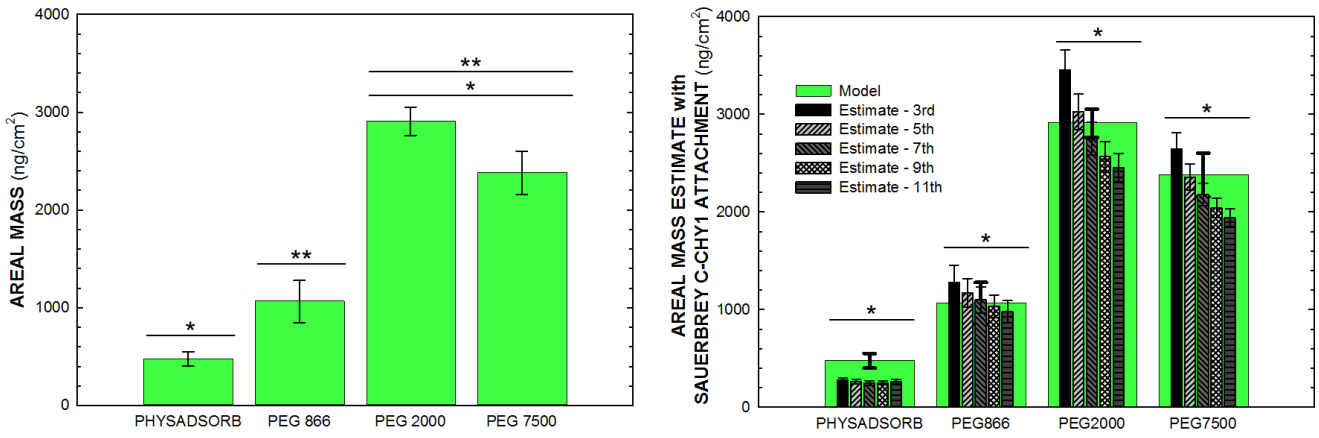


Figure 4: Areal Mass versus PEG Length Calculated using the Voigt-Kelvin Model. (a) The areal mass for each PEG size and physically-adsorbed CHY1 calculated using the Voigt-Kelvin model; (b) the Voigt-Kelvin areal mass (green) compared with an estimated areal mass (cross-hatched bars) calculated with the Sauerbrey mass due to PEG, a 100% C-CHY1 grafting efficiency assumption, and the QCM-D crystal working surface area. Reported error is standard error with n>3 replicates. For (a), one-way ANOVA demonstrated significance between the PHYSADSORB control and PEG 2000 or PEG 7500 (*) using a post-hoc Dunnett Test ($p<0.05$) and was also observed with a post-hoc Tukey Test ($p<0.05$) between PEG 866 and PEG 2000 or PEG 7500 (**), but not between PEG 2000 and PEG 7500. For the cross-hatched bars in (b), two-way ANOVA demonstrated significance ($p<0.05$) between all data sets. No significance was seen between the model and estimated values in (b).

4.3.4 The Longest Tether Length Demonstrates the Highest Bacterial Mortality and is Independent of Bacteria Type. For *E. coli*, the highest activity was achieved with the longest spacer length used, PEG 7500 (Figure 5). For the shortest spacer PEG 866, 46 ± 2.3% mortality of *E. coli* was achieved. Similarly, the highest activity of C-CHY1 against *S. aureus* was achieved with the longest spacer length, PEG 7500, at 64 ± 4.5% mortality (Figure 5). The physically-adsorbed CHY1 (Figure 5) caused 52.3 ± 1.2% and 56.7 ± 1.1% mortalities of *E. coli* and *S. aureus*, respectively.

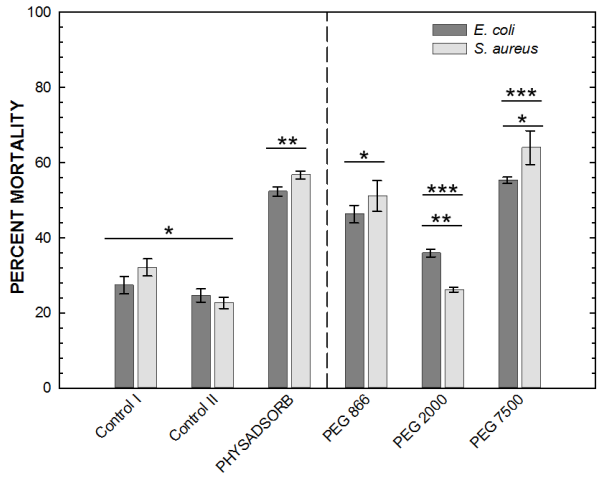


Figure 5: Average Percent Mortality of *E. coli* and *S. aureus* on Functionalized Surfaces. The average percent mortality of *E. coli* (dark bars) and *S. aureus* (light bars) was calculated after capturing fluorescent live/dead images of the surfaces *ex situ*. C-CHY1 tethered with PEG 866, 2000 and 7500 are shown to the right of the dividing line and are compared with several controls to the left of the dividing line: a bare SiO_2 surface only (control I), an APTMS-functionalized SiO_2 surface only (control II), and physically-adsorbed CHY1 (PHYSADSORB). Reported error is the standard error, corrected for sample size by dividing standard deviation by the square root of the number of replicates, n>3. Two-way ANOVA was performed. A post-hoc Dunnett Test demonstrated significance ($p<0.05$) between controls I and II and PEG 866 and PEG 7500 (*) as well as PHYSADSORB and PEG 2000 (**). A post-hoc Tukey Test also demonstrated significance ($p<0.05$) between PEG 2000 and PEG 7500 (***) within the sample set.

4.3.5 Control Experiments Demonstrate a Baseline of Approximately 25% Mortality. To ensure that the observed killing was due to peptide activity alone, experiments were done on bare, APTMS-only, and APTMS plus PEG 866, 2000, or 7500 surfaces as control experiments. For n=3 replicates of each type of control, an average baseline bacterial mortality of *E. coli* and *S. aureus* was found to be 26.1% and 24.1%, respectively.

No significant deviations from these values were observed between the different control types (supplemental data).

4.4 Discussion

AMPs that are covalently tethered with PEG provide a potential solution to preventing medical device-associated infection due to their broad spectrum activity, low likelihood of bacterial resistance, and antimicrobial activity [27, 30, 39]. Several studies have examined the antimicrobial activity of tethered AMPs, but there is still debate on the influence of tether length, AMP surface density, and AMP flexibility on tethered antimicrobial activity [24-36, 40]. We used QCM-D to calculate thickness and density of a tethered AMP, C-CHY1, with three different PEG tether lengths, and related these properties to antimicrobial activity, thus developing a mechanistic understanding of tethered AMP mechanisms.

4.4.1 Proposed Mechanism for Tethered C-CHY1 Activity

In solution, CHY1 has been shown to act against bacterial membranes through pore formation, as demonstrated by QCM-D and other techniques [49-51]. We hypothesized that tethering may cause C-CHY1 to deviate from its native mechanism in solution. Tethered C-CHY1 activity was not seen to be dependent on bacteria type, but was influenced by tether length and peptide surface density, as similar trends with respect to PEG properties were seen for *E. coli* and *S. aureus*. This agrees with the identical MICs determined of C-CHY1 against both strains (supplemental data). Thus, we propose that the mechanisms of action do not differ between strains. The ability of QCM-D to characterize layer thickness and density in a non-destructive manner allowed us to couple these results with antimicrobial assays, and thus determine C-CHY1 mechanisms of antimicrobial action for each tether length (Figure 6).

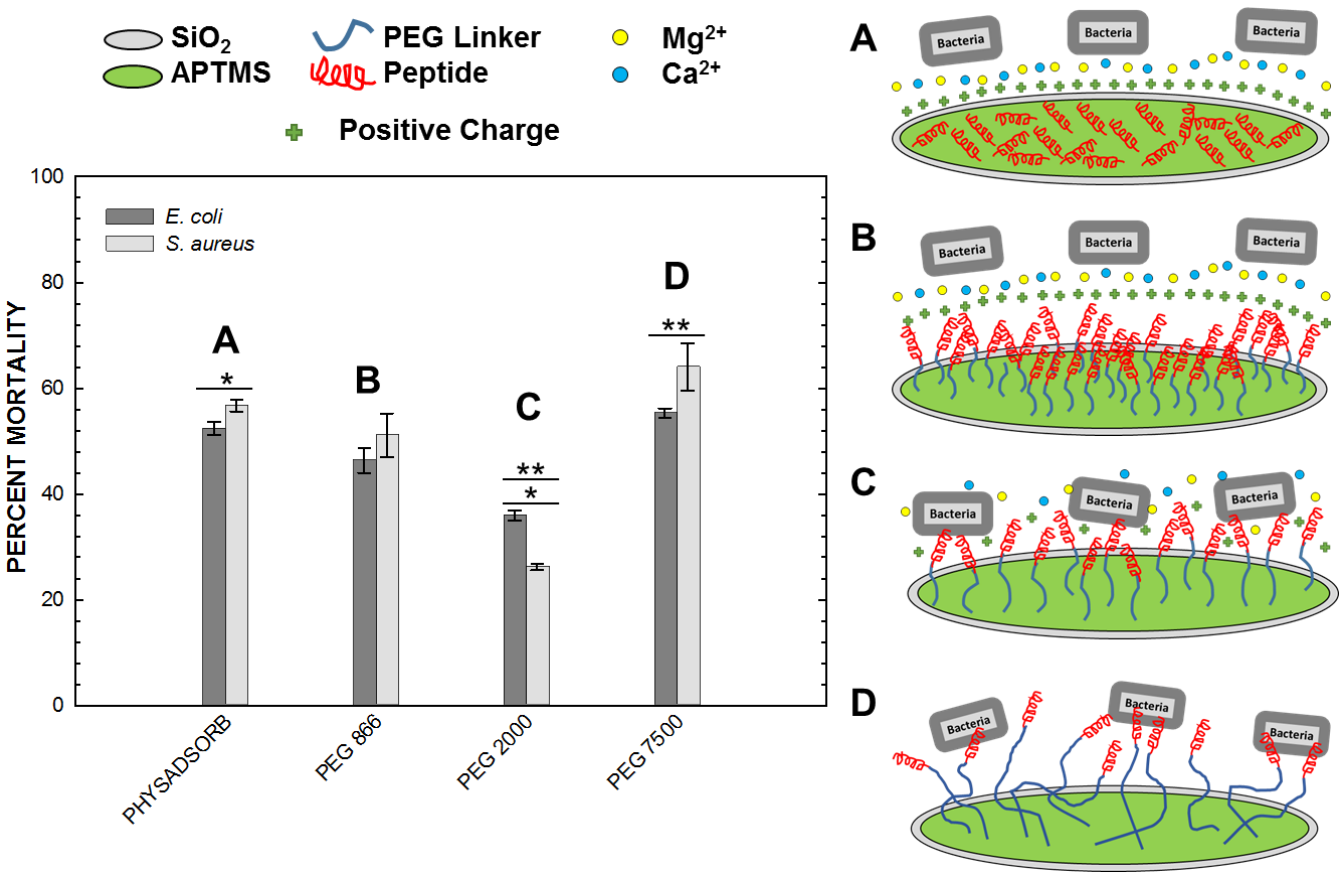


Figure 6: Mechanism of Antimicrobial Activity of Tethered Chrysophsin-1 via Different PEG Linker Lengths. The average percent mortality of *E. coli* (dark bars) and *S. aureus* (light bars) for peptide that is (A) physically-adsorbed CHY1, or C-CHY1 tethered with (B) PEG 866; (C) PEG 2000; and (D) PEG 7500. Reported error is the standard error, corrected for sample size by dividing standard deviation by the square root of the number of replicates, n>3. Two-way ANOVA and post-hoc Tukey Test ($p<0.05$) demonstrates significance between PHYSADSORB and PEG 2000 (*) and not PEG 866 or PEG 7500, and between PEG 2000 and PEG 7500 (**).

4.4.2 Proposed mechanism for Physically-adsorbed peptide, CHY1

For the physically-adsorbed CHY1, in which there was no spacer, greater than 50% bacteria mortality was observed. In this case, a high local charge density could cause an alternative mechanism of antimicrobial activity to pore formation, as seen in other studies, involving the displacement of positive cations from the membranes of both *E. coli* and *S. aureus* [41-43] (Figure 6A). Haynie *et al.* [41] observed that AMPs tethered

to short spacers (2-6 carbons) had greater than 99.9% activity, yet with the short spacers only outer membrane interactions could be achieved by the AMP. Thus a novel mechanism for AMP activity was proposed, similar to that of other polycations, membrane susceptibility caused by divalent cations (primarily Mg^{2+} and Ca^{2+}) [41-43]. Kulger *et al*. later confirmed this mechanism and demonstrated that there is a minimum threshold of this charge density before bactericidal activity begins [42]. They showed that highly cationic substrates, such physically-adsorbed or bound AMPs, promote electrostatic compensation leading to the loss of positive cations from the bacterial membrane and cell death. Lastly, Lienkamp *et al*. [43] found that by changing the local charge density they could control the antimicrobial activity of the substrate via this destabilization mechanism.

Here, we observe a discrepancy in mortality compared to our previous study (52.3 ± 1.2% vs. 19 ± 1%) [28]. Our calculated areal mass suggests that there is 5 times more CHY1 on the surface than what was observed previously (Table 1) [28]. It is possible that the lower density of CHY1 observed by Ivanov *et al.* [28] is close to the minimum charge density threshold for activity as suggested by Kügler *et al.* [42]. The higher mortality observed here follows the trends observed by Madkour *et al*. Lienkamp *et al.* [43], where increasing charge density increases antimicrobial activity.

A parallel, yet secondary mechanism, physically-adsorbed CHY1 peptides may leach into solution because of their unstable, non-covalent attachment to the surface, allowing them to adopt their native pore-forming mechanism in the presence of bacteria. However, desorption of the AMP from the surface is not ideal for clinical applications of AMPs, as it can lead to cytotoxicity and short half-life of the peptide *in vivo* [9, 10, 39].

4.4.3 Proposed mechanism for C-CHY1 tethered with PEG 866

For the shortest tethered system with PEG 866, its thickness is only 6.49 ± 1.14 nm which does not physically allow the peptide to span the *E. coli* or *S. aureus* membranes, which are 23 and 80 nm thick, respectively [28, 52]. Despite this, there is still antimicrobial activity, suggesting an alternative mechanism to pore formation similar to what was proposed by Kügler *et al.* [42] and Lienkamp *et al.* [43] (Figure 6B). This

mechanism is also consistent with our QCM-D findings. The high density of PEG 866 molecules (Figure 2; supplemental data) leads to a high density of C-CHY1 on the surface and thus a high local charge density. Higher charge densities cause stronger ionic displacement in bacterial membranes; thus, we propose that the mechanism of PEG 866-tethered C-CHY1 is membrane destabilization due to displacement of positive cations from bacterial membranes, ultimately leading to cell death [42, 43] (Figure 6B).

There are some differences in the present study compared to some of our previous work, where we reported that PEG 866-grafted C-CHY1 caused a higher mortality against *E. coli* (82 ± 11% vs. 46 ± 2.3%). Key differences are that our prior study was based on fewer replicates (n=3 vs. n=6), and changes were made in the bacterial preparation process. For example, upon repeating bacterial growth curves, we found that the OD_{600} = 1.0 used in the previous study corresponds to the stationary phase of *E. coli* growth. The present study used bacteria growing in the exponential phase, and we know that small changes in bacterial growth phase could markedly change the outcome of the experiment, especially in terms of viability.

4.4.4 Proposed mechanism for C-CHY1 tethered with PEG 2000

C-CHY1 tethered to PEG 2000 demonstrated the lowest ability to kill either bacterial strain, suggesting that the PEG tethering interferes with antimicrobial activity. The thickness of this system, 17.6 ± 1.3 nm, suggests that the spacer is 98% extended but is still not long enough to fully penetrate either membrane [28, 52]. Partial insertion of C-CHY1 into the membrane is possible, but this would limit the extent to which lysis and cell death occur. Despite the inability of PEG 2000-tethered C-CHY1 to adequately form pores, some activity still results, likely from a different mechanism. The comparatively low bacterial mortality of C-CHY1 with PEG 2000 versus PEG 866 tethers is due to lower charge density of C-CHY1 on the surface, since bactericidal activity has been shown to increase with increasing charge density [43] (Figure 6C; supplemental data). The lower charge density is due to steric hindrance between grafted PEG 2000 molecules leading to fewer binding sites available for C-CHY1 (Figure 2). The proposed mechanism of action of PEG 2000 against both bacterial strains is the ion displacement mechanism [42, 43].

4.4.5 Proposed mechanism for C-CHY1 tethered with PEG 7500

C-CHY1 tethered to PEG 7500 exhibits the most efficient bactericidal activity against both strains of bacteria, consistent with our hypothesis that longer tethers demonstrate higher activity [26, 27, 39]. Despite the lowest amount of PEG on the surface, lowest binding site availability, and the least peptide grafted onto the surface (Figure 2; Figure 4; supplemental data), its long length provides enough thickness (Figure 3) to fully penetrate the bacterial membranes and form pores, similar to how the peptide acts in solution [49, 50]. This thickness, however, is only 38% of its maximum theoretical extension, suggesting that the PEG chains in the system are entangled when there are no bacteria present [53]. It is possible that C-CHY1 extension changes in the presence of bacteria due to changes in local charge allowing it to aggregate and then form pores. Many studies suggest that pore formation does not begin until a sufficient peptide-to-lipid ratio of aggregated peptides on the surface of the membrane is achieved [51]. Thus, the proposed mechanism of action is pore formation followed by lysis and cell death, thus allowing for the highest activity across all tether lengths (Figure 6D).

4.5 QCM-D as a Tool for the Study of Tethered AMPs

Based on QCM-D data, layer thickness and grafting density were calculated using the Voigt-Kelvin viscoelastic model. As expected, thickness of the layers was positively correlated with increasing tether length; PEG 866 and 7500 demonstrated the shortest and longest layer thicknesses, respectively. The thickness of the PEG 866 and peptide layer at 6.6 nm, similar to the 9.5 nm found in a previous study [28]. Interestingly, the layer with PEG 7500 is only 38% extended compared with layers formed with PEG 866 and PEG 2000, which are both greater than 75% extended. This is consistent with the formation of a “mushroom like monolayer” of PEG 7500, where the chains are tangled [48]. Using a similar silanization procedure on SiO_2, Yang *et al.* [53] found that PEG MW 750 molecules extended ~85% of their theoretical maximum thickness and that larger PEG MW 5000 molecules only extended ~25%. Atomic force microscopy studies confirmed the formation of “mushroom like monolayers” as the long chains interpenetrated [53]. These results are comparable to what

we found in this study; larger PEG molecules interpenetrate due to the favorable thermodynamics of entanglement and self interaction [53].

In this study, the PEG and C-CHY1 layers were assembled using a "grafting to" technique [48]. "Grafting to" techniques allow strict control of tether length by the addition of whole PEG chains versus "grafting from" techniques, where the addition of single monomers at a time leaves a relatively heterogeneous brush in terms of length [48]. Thus, grafting the longest PEG 7500 tether leads to steric hindrance that limits not only PEG grafting to the surface but also the number of binding sites available for the peptide [48, 54], important factors for tethered AMP activity [39].

Similarly, we expected that areal mass would increase with increasing tether length. Sauerbrey calculations suggest that fewer PEG molecules attach as tether length increases, implying that there is also increased spacing between them. With increased spacing, more water molecules may be trapped within PEG chains and thus, more contributed mass. Indeed, Voigt-Kelvin modeling of raw QCM-D data demonstrated higher areal masses for both PEG 2000 and PEG 7500 than PEG 866 (Table 1). Inconsistent with our expectations; however, was that the areal mass due to PEG 2000 was higher than that of PEG 7500. Even though there are considerably more water molecules associated with one PEG 7500 molecule compared with one PEG 2000 molecule (Figure 7), there are significantly more PEG 2000 molecules (a; 42 ng total) compared to PEG 7500 (b; 2 ng total) leading to a higher areal mass. It is unlikely that loose C-CHY1 molecules within the PEG chains are contributing to the reported areal mass because these are washed away with the subsequent buffer rinse. Further, loose C-CHY1 molecules would be hindered by the dense PEG 2000 packing (Figure 7a) or the tangled mushroom-like structure of PEG 7500 chains (Figure 7b).

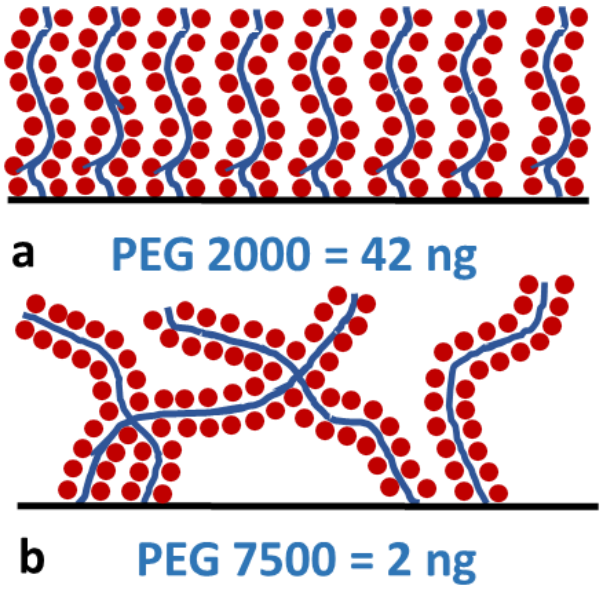


Figure 7: The Association of Water Molecules with (a) PEG 2000 and (b) PEG 7500 Layers Leading to Differences in Areal Mass. More water molecules per chain are associated with PEG 7500; however, there are many more PEG 2000 molecules on the surface leading to a higher mass per area.

CHY1 introduction did not cause significant changes in ΔD from the baseline value, suggesting that there were no changes in film flexibility or hydration as a result of peptide adsorption. Layer thickness and density of the physically-adsorbed CHY1 were also calculated for comparison to our past work [28]. The calculated thickness here is 2.5 nm, but it is known that when in alpha-helical form, CHY1 is 3.5 nm long and its helix is 1.2 nm in diameter [28]. Thus, a thickness between these values suggests that physically-adsorbed peptides are randomly oriented on the surface and are likely not aggregated [25]. The areal mass calculated was 475 ± 75 ng/cm^2 (Table 1), 80% higher than the previously-reported 93.5 ng/cm^2 (Table 1) [28]. This discrepancy not only suggests 5 times more CHY1 on the surface, but also considerably higher local charge density with each CHY1 having a +6 charge. This explains the difference seen in *E. coli* mortality reported between the two studies, as discussed above.

A considerable advantage of the QCM-D technique is its ability to allow layer thickness and density to be coupled to antimicrobial activity. With QCM-D we were able to differentiate the pore-forming and ion displacement mechanisms by measuring thickness and surface density in real-time and relating them to antimicrobial activity. Other studies could only do this using multiple techniques. This study demonstrates the usefulness of QCM-D as a non-destructive tool for characterizing tethered AMPs and determining their antimicrobial mechanisms. We also demonstrated new insight into the role of tether length in determining AMP activity against gram negative and gram positive bacteria.

4.6 Conclusions

QCM-D was used to study covalently-tethered AMP C-CHY1 via PEG molecules of three different lengths (PEG 866, 2000, and 7500), with the goal of understanding how tether length and grafting density change mechanisms leading to antimicrobial action against *E. coli* and *S. aureus*. QCM-D allowed us to measure density and thickness and couple these properties with antimicrobial activity. We proposed a mechanism of action for each tether length. The longest tether allowed proper AMP orientation and its native pore-forming mechanism leading to the highest activity; short tethers led to comparatively denser grafting, forcing a non-native, yet still potent ion displacement mechanism. C-CHY1 peptides tethered with medium tethers could not wholly adopt either mechanism and thus were less effective. These results allow more rational designs of tethered AMPs for clinical applications and demonstrate the feasibility for using QCM-D to study their mechanisms.

This work is published in **Lozeau, L.D.**, **T.E. Alexander**, and T.A. Camesano, *Proposed mechanisms of tethered antimicrobial peptide chrysophsin-1 as a function of tether length using QCM-D.* The Journal of Physical Chemistry B, 2015. **119**(41): p. 13142-13151.

REFERENCES

1. Centers for Disease Control and Prevention, *Antibiotic resistance threats in the United States, 2013. Atlanta: CDC; 2013*. 2014.
2. Hancock, R.E. and R.J.T.i.b. Lehrer, *Cationic peptides: a new source of antibiotics.* 1998. **16**(2): p. 82-88.
3. Hancock, R.E.J.T.L.i.d., *Cationic peptides: effectors in innate immunity and novel antimicrobials.* 2001. **1**(3): p. 156-164.
4. Jenssen, H., P. Hamill, and R.E.J.C.m.r. Hancock, *Peptide antimicrobial agents.* 2006. **19**(3): p. 491-511.
5. Hancock, R.E. and G.J.T.i.m. Diamond, *The role of cationic antimicrobial peptides in innate host defences.* 2000. **8**(9): p. 402-410.
6. Hancock, R.E. and H.-G. Sahl, *Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies.* Nature biotechnology, 2006. **24**(12): p. 1551.
7. Nizet, V., *Antimicrobial peptide resistance mechanisms of human bacterial pathogens.* Current issues in molecular biology, 2006. **8**(1): p. 11.
8. Li, P., et al., *Antimicrobial macromolecules: synthesis methods and future applications.* Rsc Advances, 2012. **2**(10): p. 4031-4044.
9. Marr, A.K., W.J. Gooderham, and R.E. Hancock, *Antibacterial peptides for therapeutic use: obstacles and realistic outlook.* Current opinion in pharmacology, 2006. **6**(5): p. 468-472.
10. Reddy, K., R. Yedery, and C. Aranha, *Antimicrobial peptides: premises and promises.* International journal of antimicrobial agents, 2004. **24**(6): p. 536-547.
11. Brogden, K.A., *Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?* Nature reviews microbiology, 2005. **3**(3): p. 238.

12. Steiner, H., D. Andreu, and R.B. Merrifield, *Binding and action of cecropin and cecropin analogues: antibacterial peptides from insects.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 1988. **939**(2): p. 260-266.

13. Pouny, Y., et al., *Interaction of antimicrobial dermaseptin and its fluorescently labeled analogs with phospholipid membranes.* Biochemistry, 1992. **31**(49): p. 12416-12423.

14. Powers, J.-P.S. and R.E. Hancock, *The relationship between peptide structure and antibacterial activity.* Peptides, 2003. **24**(11): p. 1681-1691.

15. Zasloff, M., *Antimicrobial peptides of multicellular organisms.* nature, 2002. **415**(6870): p. 389.

16. Duplantier, A.J. and M.L. van Hoek, *The human cathelicidin antimicrobial peptide LL-37 as a potential treatment for polymicrobial infected wounds.* Frontiers in immunology, 2013. **4**: p. 143.

17. Braff, M.H., et al., *Keratinocyte production of cathelicidin provides direct activity against bacterial skin pathogens.* Infection and immunity, 2005. **73**(10): p. 6771-6781.

18. Maher, S. and S. McClean, *Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro.* Biochemical pharmacology, 2006. **71**(9): p. 1289-1298.

19. Kazemzadeh-Narbat, M., et al., *Antimicrobial peptides on calcium phosphate-coated titanium for the prevention of implant-associated infections.* Biomaterials, 2010. **31**(36): p. 9519-9526.

20. Darouiche, R.O., *Treatment of infections associated with surgical implants.* New England Journal of Medicine, 2004. **350**(14): p. 1422-1429.

21. Arciola, C.R., et al., *Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials.* Biomaterials, 2012. **33**(26): p. 5967-5982.

22. Banerjee, I., R.C. Pangule, and R.S. Kane, *Antifouling coatings: recent developments in the design of surfaces that prevent fouling by proteins, bacteria, and marine organisms.* Advanced materials, 2011. **23**(6): p. 690-718.

23. Batoni, G., et al., *Use of antimicrobial peptides against microbial biofilms: advantages and limits*. Current medicinal chemistry, 2011. **18**(2): p. 256-279.

24. Hickok, N.J. and I.M. Shapiro, *Immobilized antibiotics to prevent orthopaedic implant infections.* Advanced drug delivery reviews, 2012. **64**(12): p. 1165-1176.

25. Gabriel, M., et al., *Preparation of LL-37-grafted titanium surfaces with bactericidal activity.* Bioconjugate chemistry, 2006. **17**(2): p. 548-550.

26. Bagheri, M., *Cationic Antimicrobial Peptides: Thermodynamic Characterization of Peptide-Lipid Interactions and Biological Efficacy of Surface-Tethered Peptides*. 2010, Freie Universität Berlin.

27. Bagheri, M., M. Beyermann, and M. Dathe, *Immobilization reduces the activity of surface-bound cationic antimicrobial peptides with no influence upon the activity spectrum.* Antimicrobial agents and chemotherapy, 2009. **53**(3): p. 1132-1141.

28. Ivanov, I.E., et al., *Creating Antibacterial Surfaces with the Peptide Chrysophsin-1*. ACS Applied Materials & Interfaces, 2012. **4**(11): p. 5891-5897.

29. Blin, T., et al., *Bactericidal microparticles decorated by an antimicrobial peptide for the easy disinfection of sensitive aqueous solutions.* Biomacromolecules, 2011. **12**(4): p. 1259-1264.

30. Lu, S., *Immobilization of antimicrobial peptides onto titanium surfaces*. 2009, University of British Columbia.

31. Noimark, S., et al., *The role of surfaces in catheter-associated infections.* Chemical Society Reviews, 2009. **38**(12): p. 3435-3448.

32. Shukla, A., et al., *Controlling the release of peptide antimicrobial agents from surfaces.* Biomaterials, 2010. **31**(8): p. 2348-2357.

33. Gershkovich, E., *Zuckerberg Institute for Water Research*. 2009, Citeseer.

34. Glinel, K., et al., *Antibacterial surfaces developed from bio-inspired approaches.* Acta Biomaterialia, 2012. **8**(5): p. 1670-1684.

35. Gao, G., et al., *Biomembrane Interactions Reveal the Mechanism of Action of Surface-Immobilized Host Defense IDR-1010 Peptide.* Chemistry & Biology, 2012. **19**(2): p. 199-209.

36. Gao, G., et al., *Antibacterial surfaces based on polymer brushes: investigation on the influence of brush properties on antimicrobial peptide immobilization and antimicrobial activity.* Biomacromolecules, 2011. **12**(10): p. 3715-3727.

37. Hilpert, K., *Identifying novel antimicrobial peptides with therapeutic potential against multidrug-resistant bacteria by using the SPOT synthesis.* Mini-Reviews in Organic Chemistry, 2011. **8**(2): p. 157-163.

38. Appendini, P. and J. Hotchkiss, *Surface modification of poly (styrene) by the attachment of an antimicrobial peptide.* Journal of applied polymer science, 2001. **81**(3): p. 609-616.

39. Onaizi, S.A. and S.S. Leong, *Tethering antimicrobial peptides: current status and potential challenges.* Biotechnology advances, 2011. **29**(1): p. 67-74.

40. Brogden, N.K. and K.A. Brogden, *Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals?* International journal of antimicrobial agents, 2011. **38**(3): p. 217-225.

41. Haynie, S.L., G.A. Crum, and B.A. Doele, *Antimicrobial activities of amphiphilic peptides covalently bonded to a water-insoluble resin.* Antimicrobial Agents and Chemotherapy, 1995. **39**(2): p. 301-307.

42. Kügler, R., O. Bouloussa, and F. Rondelez, *Evidence of a charge-density threshold for optimum efficiency of biocidal cationic surfaces.* Microbiology, 2005. **151**(5): p. 1341-1348.

43. Lienkamp, K., et al., *Antimicrobial Polymers Prepared by Ring-Opening Metathesis Polymerization: Manipulating Antimicrobial Properties by Organic Counterion and Charge Density Variation.* Chemistry–A European Journal, 2009. **15**(43): p. 11715-11722.

44. Porcelli, F., et al., *NMR structure of the cathelicidin-derived human antimicrobial peptide LL-37 in dodecylphosphocholine micelles.* Biochemistry, 2008. **47**(20): p. 5565-5572.

45. Wang, K.F., R. Nagarajan, and T.A. Camesano, *Antimicrobial peptide alamethicin insertion into lipid bilayer: a QCM-D exploration.* Colloids and Surfaces B: Biointerfaces, 2014. **116**: p. 472-481.

46. Wiegand, I., K. Hilpert, and R.E. Hancock, *Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances.* Nature protocols, 2008. **3**(2): p. 163.

47. Oelmeier, S.A., F. Dismer, and J. Hubbuch, *Molecular dynamics simulations on aqueous two-phase systems-Single PEG-molecules in solution.* BMC biophysics, 2012. **5**(1): p. 14.

48. Minko, S., *Grafting on solid surfaces:"Grafting to" and "grafting from" methods*, in *Polymer surfaces and interfaces*. 2008, Springer. p. 215-234.

49. Iijima, N., et al., *Purification and characterization of three isoforms of chrysophsin, a novel antimicrobial peptide in the gills of the red sea bream, Chrysophrys major.* European Journal of Biochemistry, 2003. **270**(4): p. 675-686.

50. Mason, A.J., et al., *Membrane interaction of chrysophsin-1, a histidine-rich antimicrobial peptide from red sea bream.* Biochemistry, 2007. **46**(51): p. 15175-15187.

51. Wang, K.F., et al., *Characterization of supported lipid bilayer disruption by chrysophsin-3 using QCM-D.* The Journal of Physical Chemistry B, 2011. **115**(51): p. 15228-15235.

52. Ayman, E.-H. and S.H. Jayaram. *Effect of biological cell size and shape on killing efficiency of pulsed electric field*. in *2008 IEEE International Conference on Dielectric Liquids*. 2008. IEEE.

53. Yang, Z., J.A. Galloway, and H. Yu, *Protein interactions with poly (ethylene glycol) self-assembled monolayers on glass substrates: diffusion and adsorption.* Langmuir, 1999. **15**(24): p. 8405-8411.

54. Wang, W., et al., *Effect of a novel antimicrobial peptide chrysophsin-1 on oral pathogens and Streptococcus mutans biofilms*. Peptides, 2012. **33**(2): p. 212-219.

4.7 Supporting Information

Minimum Inhibitory Concentration (MIC) Assay. The broth dilution procedure by Weigand *et al.* followed for CHY1 and C-CHY1 against common pathogenic microbes[46]. The MIC was determined against *E. coli* (ATCC 33694), *Pseudomonas aeruginosa* (ATCC 29260), *S. aureus* (ATCC 43866) and *Staphylococcus epidermidis* (ATCC 49461) for CHY1 to be between 0.14 and 8.65 μM and for C-CHY1 to be between 2.25 and 5.60 μM, respectively, against these microbes (supplemental Table 1). The MIC ranges are similar, demonstrating little effect of cysteine modification on antimicrobial activity. This is reasonable compared both to literature estimates and our previous work in solution[28,S1,S2]. Thus, C-CHY1 in solution demonstrates broad activity.

Supplemental Table 1: The Experimental MIC (μM) of CHY1 and C-CHY1 Found Using Broth Dilution Methodology.

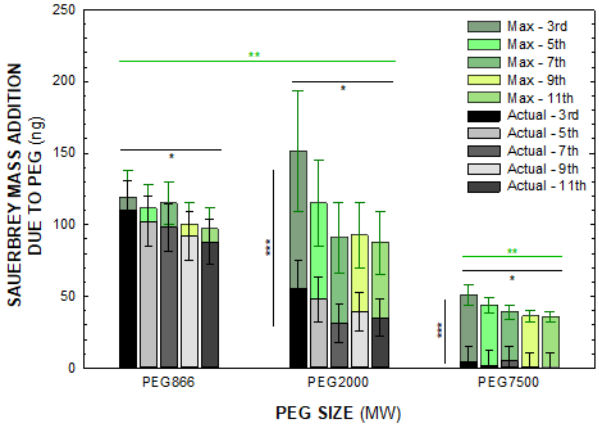
Bacteria[a]	*CHY1 (Lit.)*	*Ref.*	*CHY1 (Exp.)*	*C-CHY1 (Exp.)*
E. coli	2.23	S1	4.17	2.25
E. coli[b]	2.23	S1	< 10	< 10
S. aureus	2.23	S1	2.5	2.25
S. aureus[b]	1.4 – 4.2	S1	< 10	< 10
S. epidermidis	0.18 – 20.8	S2	8.65	2.25
P. aeruginosa	1.4 – 4.2	S2	2.5	5.60

[a]ATCC numbers: 33694, 43866, 49461, and 29260 for each microbe type, respectively

[b]Bactericidal activity measured by Ivanov and colleagues[28]

Sauerbrey Mass Attachment Due to PEG and C-CHY1 Molecules. The mass attachment due to PEG (supplemental Figure 1) and C-CHY1 (supplemental Figure 2) molecules was calculated using the Sauerbrey equation assuming rigid, or a low ΔD film, where mass addition is inversely related to Δ*f*.

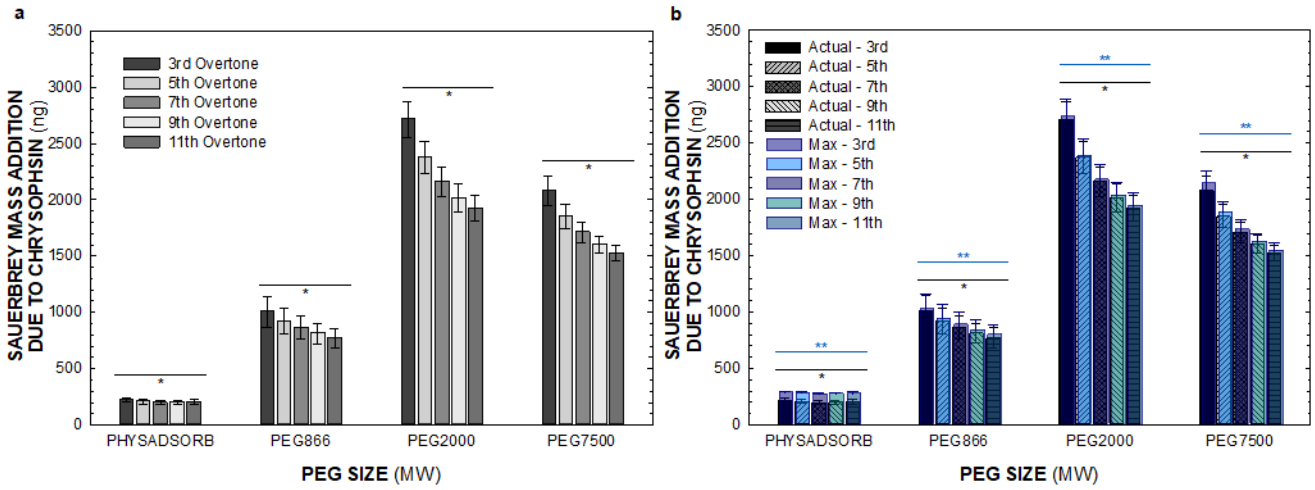
In supplemental Figure 1, the actual PEG attachment (grey bars) represents a Δ*f* found by subtracting the *f* at the point of PEG flow from the *f* at the point of C-CHY1 flow. The maximum PEG attachment (green bars) represents a Δ*f* found by subtracting the *f* at the point of PEG flow from the lowest value of *f* during the time period from PEG flow to C-CHY1 flow. The differences between the two values for each PEG shows a grafting efficiency. While nearly all of the PEG 866 flown through the QCM-D attaches to the surface while much of the PEG 2000 and PEG 7500 are rinsed from the surface, likely due to steric hindrance from neighboring peptides.



Supplemental Figure 1: Maximum and Actual Mass Attachment (ng) due to PEG Flow versus PEG Size. Mass attachment was estimated for each PEG molecular weight (PEG 866, 2000 and 7500) at the 3rd through 11th overtones. Actual PEG attachment is represented in grey and maximum PEG attachment is represented in green. Reported error is standard error, corrected for sample size by dividing standard deviation by the square root of the number of replicates. Two-way ANOVA and post-hoc Tukey Tests ($p<0.05$) on each data set were performed. As in Figure 2, significance (*) between all three groups was found for the actual attachment data (grey). Significance between PEG 866 or PEG 2000 and PEG 7500 was demonstrated (**),

but not between PEG 866 and PEG 2000 in the case of maximum PEG attachment (green). A difference was also observed between maximum and actual attachment values for PEG 2000 and PEG 7500 (***) but not for PEG 866, using the averages between the 3rd through 11th overtones.

In supplemental Figure 2, the actual peptide attachment (grey bars in (a), cross-hatched in (b)) represents a Δ*f* found by subtracting the *f* at the point of peptide flow from the *f* at the point of bacteria flow. The maximum peptide attachment (blue bars) represents a Δ*f* found by subtracting the *f* at the point of peptide flow from the lowest value of *f* during the time period from peptide flow to bacteria flow. The differences between the two values for the peptide demonstrates a grafting efficiency. There was no significant differences between the maximum and actual peptide attachment, thus demonstrating that nearly all of the peptide that contacted the PEGylated surface attached strongly.

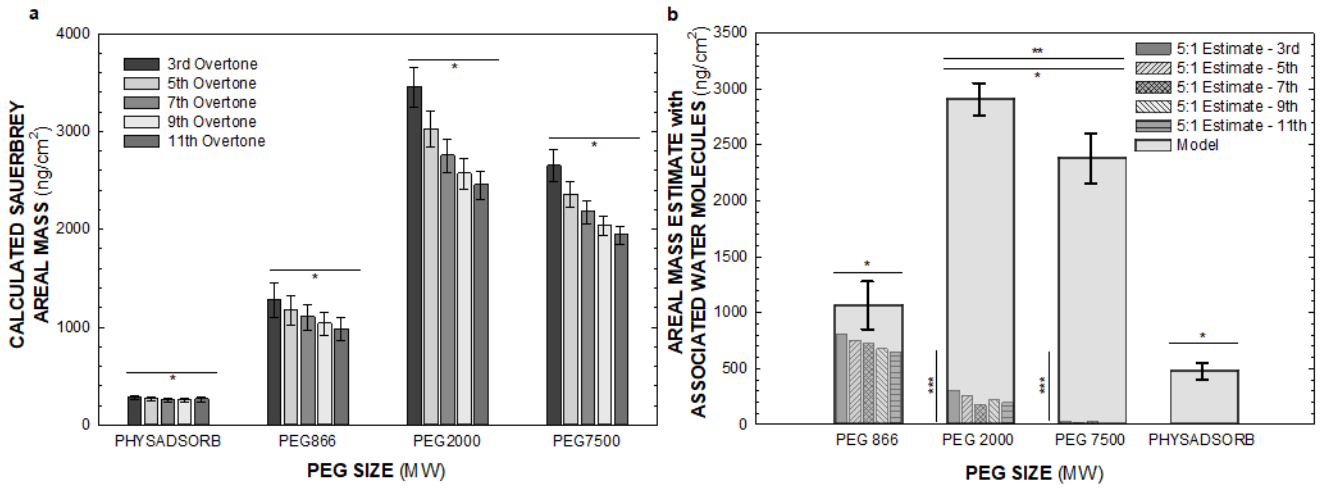


Supplemental Figure 2: Mass Attachment (ng) due to Chrysophsin-1 Flow versus PEG Size. Mass attachment was estimated for each PEG (PEG MW 866, 2000 and 7500) and the physically-adsorbed control (PHYSADSORB), n>3, at the 3rd through 11th overtones. The actual attachment is shown in (a) and is cross-hatched in (b) for its comparison with maximum attachment (blue). Reported error is standard error, corrected for sample size by dividing standard deviation by the square root of the number of replicates, n>3. Two-way ANOVA was performed. Significance ($p<0.05$) between PHYSADSORB, PEG 866, PEG 2000 and PEG

7500 for both actual (*) and maximum (**) data sets was found using both pairwise post-hoc Tukey Tests and Dunnett Tests. Within the groups, there was no significance between actual and maximum data sets.

Estimations of Peptide Areal Mass. The total system mass in nanograms, including PEG Tethers and C-CHY1, was first estimated for all overtones and PEG lengths, using the Sauerbrey attachment data for comparison to the Voigt-Kelvin model (supplemental Figure 3a). Here, the Δf used was found by subtracting f at the start of PEG flow from the f at the start of bacteria flow.

The effect of the associated water molecules is shown in supplemental Figure 3b, where a 5:1 ratio of water molecules associated with each PEG monomer (12, 38 and 163 monomers for PEG 866, 2000, and 7500 respectively) was used as a baseline to calculate an estimated attachment of mass due to water. This 5:1 ratio is commonly found with PEG chains, and was chosen based on literature[47]. PEG mass was calculated using Sauerbrey, and C-CHY1 mass was calculated using the assumption of 100% peptide grafting efficiency (PEG to C-CHY1 ratio = 1) to the calculated amount of PEG. Then, the total system mass was calculated by adding the masses due to PEG tether molecules, C-CHY1, and water in the system for all overtones. These values were compared to the model areal mass (large grey bars), calculated by multiplying the brush layer thickness (nm) by the value of density (ng/nm^3), both found using the Voigt-Kelvin extended viscoelastic model.



Supplemental Figure 3 (a): Areal Mass Calculated using Sauerbrey Chrysophsin-1 Attachment Estimates. Areal mass (nm/cm^2) was calculated using the Sauerbrey mass divided by the available QCM-D crystal working surface area (a) for each PEG molecular weight (PEG 866, 2000 and 7500) and the physically-adsorbed control (PHYSADSORB) at the 3^{rd} through 11^{th} overtones. **(b):** Model versus Estimated Areal Mass using PEG Chain and Water Association to Calculate Total Mass. All overtones (3^{rd} through 11^{th}) were used to model each data set with the Voigt-Kelvin model (large grey bars). This is compared to an estimated areal mass (small cross-hatched bars each representing an overtone 3^{rd} through 11^{th}), calculated by adding the estimated masses of PEG, C-CHY1, and water molecules associated with PEG monomers in a 5:1 ratio[47]. All reported error is standard error, corrected for sample size by dividing standard deviation by the square root of the number of replicates, $n>3$. For (a), two-way ANOVA was performed. Significance (*) between all PEG samples and the PHYSADSORB control ($p<0.05$) was found using a post-hoc Dunnett Test. For (b), one-way ANOVA was performed. Significance was observed between the PHYSADSORB control and PEG 2000 or PEG 7500 (*) using a post-hoc Dunnett Test ($p<0.05$) and was also observed with a post-hoc Tukey Test ($p<0.05$) between PEG 866 and PEG 2000 or PEG 7500 (**), but not between PEG 2000 and PEG 7500 for the model bars. There was a significant difference ($p<0.05$) between the estimated values and model values for PEG 2000 and PEG 7500 only (***).

Sauerbrey estimations for all samples were comparable to those found using the Voigt-Kelvin calculation, (a). On the contrary, the calculated total mass of the system assuming a 5:1 ratio of water molecules associated with each PEG monomer was significantly underestimated for PEG 2000 and 7500 in (b). This is because the water associated with the PEG monomers does not fill the entirety of the spacing between neighboring peptides. Thus, much more water is associated in these cases and significantly contributes to the total mass. The estimation is comparable in the case of PEG 866 molecules because of close packing as measured by low QCM-D ΔD values, meaning that most of the intercalated water molecules contributing to the total system mass are associated with the chains only.

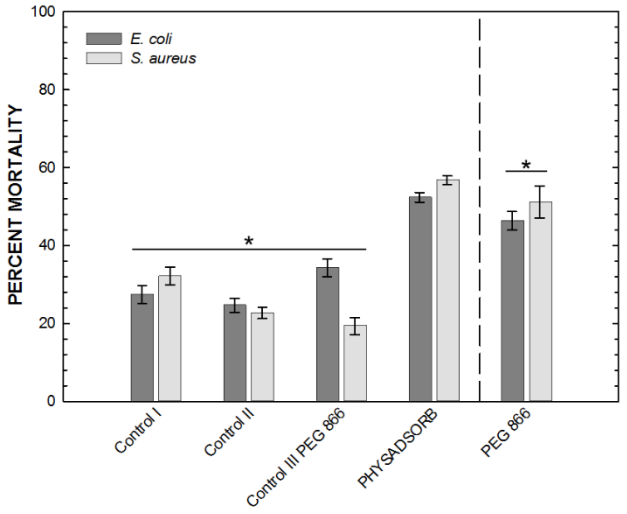
Activity of Control Surfaces. The activities of tethered C-CHY1 via PEG 866, 2000 and 7500 and physically-adsorbed CHY1 against *E. coli* and *S. aureus* was determined with *ex situ* imaging. These activities were compared with controls in supplemental Figure 4a, a bare SiO_2 surface only (control I) and an APTMS-functionalized SiO_2 surface only (control II). Additional controls included PEGylated surfaces without peptide (control III) for each PEG length. Each sample length is compared with relevant controls in supplemental Figure 4(b-d). All control surfaces show approximately 25% bacterial mortality as a baseline value.

a

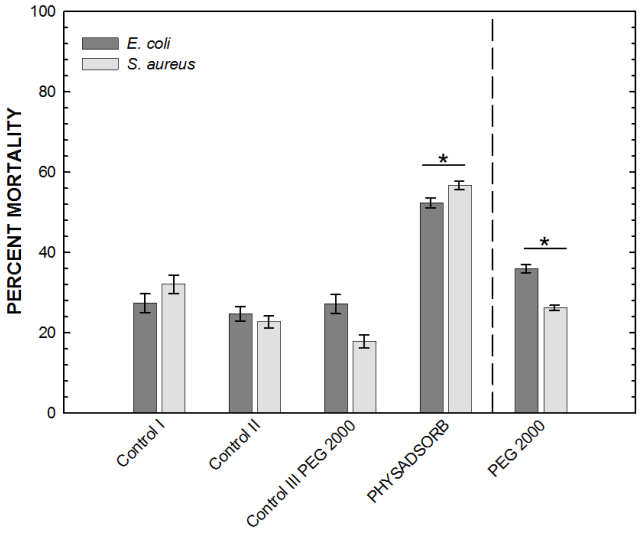
Average Killing Percent ± Standard Error

Sample	[a]*E. coli*	[a]*S. aureus*
Bare SiO_2 (Control I)	27.4 ± 2.30	32.1 ± 2.29
SiO_2 + APTMS (Control II)	24.6 ± 1.79	22.7 ± 1.48
SiO_2 + APTMS + PEG 866 (Control III)	34.3 ± 2.33	19.3 ± 2.19
SiO_2 + APTMS + PEG 2000 (Control III)	27.1 ± 2.38	17.8 ± 1.67
SiO_2 + APTMS + PEG 7500 (Control III)	17.0 ± 2.51	28.5 ± 2.42
CCHY1 / PEG 866	46.4 ± 2.34	51.1 ± 4.12
CCHY1 / PEG 2000	35.9 ± 1.00	26.2 ± 0.62
CCHY1 / PEG 7500	55.3 ± 0.87	64.0 ± 4.46
CHY1 (PHYSADSORB)	52.4 ± 1.24	56.7 ± 1.11

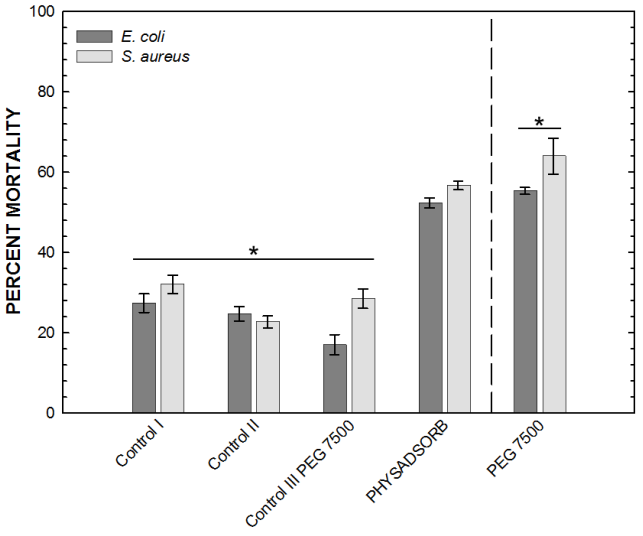
b



c



d



Supplemental Figure 4 (a): Numerical Values for the Average Bacterial Mortality versus *E. coli* and *S. aureus*. All control surfaces and samples are presented against each bacteria type. **(b-d):** Individual Average Percent Mortality of *E. coli* and *S. aureus* on Functionalized Surfaces. The average percent mortality of *E. coli* (dark bars) and *S. aureus* (light bars) was determined when exposed to C-CHY1 tethered to a SiO_2-coated, APTMS-functionalized QCM-D crystal surface with PEG tether molecules, molecular weights 866 (b), 2000 (c) and 7500 (d). In each plot, the sample is to the right of the dividing line, while relevant control groups are to the left. In all cases, the bacterial mortality was calculated by the number of cells stained red (dead) over the total number of cells imaged at each location on the crystal surface. All reported error is standard error, corrected for sample size by dividing standard deviation by the square root of the number of replicates, $n>3$. In (b-d), two-way ANOVA was performed. A post-hoc Dunnett Test demonstrated significance ($p<0.05$) only between controls I, II and III and PEG 866, but not the PHYSADSORB control (b). A post-hoc Dunnett Test demonstrated significance ($p<0.05$) only between the PHYSADSORB control and PEG 2000 (c). Similar to (b), a post-hoc Dunnett Test demonstrated significance ($p<0.05$) only between controls I, II and III and PEG 7500, but not the PHYSADSORB control (d).

Supplemental References

1. Fulmer, P. A.; Lundin, J. G.; Wynne, J. H. Development of Antimicrobial Peptides (AMPs) for Use in Self-Decontaminating Coatings. *ACS Appl. Mater. Interfaces*, **2010**, *2(4)*, 1266-1270.
2. Masson-Silva J. A; Diamond, G. Antimicrobial Peptides from Fish. *Pharmaceuticals*, **2014**, *7(3)*, 265-310.

Specific aim 2: Chapter 5 Role of lipopolysaccharide and lipoteichoic acid on C-CHY1 interactions with model Gram-Positive and Gram-Negative Bacterial Membranes

Abstract

Antimicrobial peptides (AMPs) are attractive as biomaterial coatings because they have broad spectrum activity against different types of microorganisms, and a low likelihood of incurring resistance due to their unique mechanisms of action (MOA). Direct action against the bacterial membrane is the most common MOA of AMPs, while some exhibit mechanisms dependent on membrane composition, peptide concentration, and environmental factors that include temperature and salt concentration. Supported lipid bilayers (SLBs) were used to study how temperature and lipid composition influence the MOA of the broadly active, cysteine-modified AMP Chrysophsin-1. SLB models of Gram-positive bacterial membranes were formed using combinations of phosphatidylcholine (PC), phosphatidylglycerol (PG), and *S. aureus*-derived lipoteichoic acid (LTA), and SLB models of Gram-negative bacterial membranes were formed using combinations of phosphatidylethanolamine (PE), PG, and *E. coli*-derived lipopolysaccharides (LPS). For the Gram-positive SLBs, temperature did not have a significant effect on the MOA, as seen in the similar trends in frequency and dissipation changes across all overtones and the same mechanistic trends in the polar plots when comparing the same SLB composition. SLB composition did have a significant effect on AMP MOA, with more C-CHY1 association due to interaction of the AMP with LTA, leading to SLB saturation as seen in the polar plots. For the Gram-negative SLBs, composition did not have a significant effect on MOA but temperature affected MOA due to changes in membrane fluidity when the peptide inserted into the membrane. One explanation is that since the glass transition temperature (Tg) of PE is 25^{o}C, then at 23 ^{o}C, the SLB is rigid. In this case, a significant energetic shift would be required to allow for additional AMP insertion, which can be seen in the change of slope of the C-CHY1 insertion step in the mechanism of action. At 37^{o}C, the membrane is more fluid and there is less of an energetic requirement for insertion. A better understanding of C-CHY1 MOA using different SLB models will allow for the more rational design of future therapeutic solutions, such as biomaterial coatings.

5.1 Introduction

Antibiotic resistance is projected to kill more people per year than cancer and cost over $100 trillion by 2050 unless new treatments become available [1]. Antimicrobial peptides (AMPs), are short, innate host defense proteins that have broad spectrum activity and the ability to kill resistant bacteria without further promoting resistance, due to their unique mechanisms of action (MOA) [2-6]. However, AMPs can be cytotoxic and ineffective in high salt environments such as blood serum and urine [3, 7-14]. Understanding the MOA of AMPs is important in order to understand the best way to design AMP-based therapeutics [15-18]. Chrysophsin-1 (CHY1), derived from the red sea bream, is an attractive AMP for clinical use, due to its broad spectrum activity against both Gram-positive and Gram-negative bacteria, and inherent salt tolerance [14, 19]. One major source of infections are those associated with medical devices which are difficult to treat due to biofilm formation and antimicrobial resistance [1, 20-29]. Prevention of device associated infections is the best strategy and modified versions of CHY1, such as a cysteine modified form (C-CHY1), can be covalently bound to a biomaterial surface as a strategy to prevent infections [2, 19].

Four major types of peptide interactions with membranes have been characterized: (1) toroidal pore, (2) barrel-stave, (3) intermittent pores, and (4) carpet mechanism [6]. Direct action against bacterial membranes via pore formation is the most common MOA [4-6]. Most pore-forming mechanisms are based on a non-specific interactions involving electrostatic attraction of positively charged AMPs with negative bacterial membranes, leading to pore formation, and membrane leaking, leading to cell death [4-6]. However, pore-forming AMPs, including C-CHY1, may exhibit different MOAs, including intracellular targets, which depend upon membrane composition, concentration, or environmental factors, such as temperature or salt concentration [2, 6, 30-34]. Understanding the mechanistic nuances of AMPs in different conditions may allow informed decisions on peptide design and applications. For example, the addition of a cysteine-residue onto Chrysophsin-1 allowed for its attachment onto surfaces via sufficiently-long flexible poly(ethylene glycol) linker molecules to allow pore formation [2]. The length of the spacer determined the effectiveness of C-CHY1 and mechanism when tethered [2]. Further, C-CHY1 is more effective against Gram-positive

bacteria, and thus would be better suited as a coating for orthopedic knees and hips, where Gram-positive bacteria are the major cause of infections [23, 24, 26, 35]. For other AMPs, a short stiff spacer is sufficient to allow for antimicrobial activity [36-40]. Peptide density is a critical factor in antimicrobial peptide efficacy and activity [2, 23, 36, 37, 40-42]. Lozeau *et al.* [32] demonstrated that for LL-37, and modified forms fCBD and cCBD LL37, peptide concentration (density) effected the MOA that the peptide exhibited. Additionally, it has been demonstrated that a sufficiently high density of AMPs can allow for a non-native charge based MOA [39, 43, 44]. The effects of density and temperature on the activity of C-CHY1 against Gram-positive *S. aureus* and Gram-negative *E. coli* were investigated. Unpublished data from our lab suggest that density did not have an effect on the efficacy of C-CHY1 but temperature did affect the activity of C-CHY1 against the Gram-positive *S. aureus*, increasing at 37^{o}C comparted to 23^{o}C (Supplementary Figures 1-4). Therefore, it is important to understand mechanism of action under different environmental conditions to make the best use of their activity [2, 30, 36, 45-52].

Supported lipid bilayers (SLBs) are commonly utilized as models to elucidate and distinguish AMP MOAs on membranes as a function of various peptide properties, such as charge, secondary structure, length, and various peptide modifications [15, 16, 32, 53-56]. SLB models often include one or two lipid components including phosphatidylcholines (PC), phosphatidylglycerols (PG), or combinations thereof [15, 16, 32, 41, 54]. These systems offer valuable insight into MOA of different AMPs [15, 16, 32, 33, 56]. More complex SLBs may be more representative, but are more difficult to form and characterize [54, 57-59]. Even simple systems offer valuable insight into the MOA of different AMPs and have been successfully used to determine the effect of peptide modifications, such as the addition of different short peptide sequences [15, 16, 32, 33, 56].

Membrane lipid composition and surface proteins also have a significant effect on MOA [30, 50, 52, 60-64]. This allows for AMP selectivity against bacterial cells over mammalian cells, since mammalian membranes have a higher percentage of zwitterionic lipids and typically have a more neutral charge

compared to bacterial membranes, which have a higher content of anionic lipids and proteins [6, 46, 47, 49, 50, 65, 66]. In Gram-positive and Gram-negative bacteria, negatively-charged molecules, lipoteichoic acid (LTA) and lipopolysaccharide (LPS), respectively, can make up 30% or more of the surface proteins of the bacteria [51, 61, 67-69].LPS and LTA carry a net negative charge and play a significant role in AMP interactions with bacterial membranes [51, 61, 63, 67-69]. The exact role of LPS and LTA on AMP interactions, may vary among bacterial strains [46, 47, 61, 63, 70-72]. However, a common strategy for bacteria to become resistant to AMPs is for the truncation or elimination of surface proteins, such as LPS [51]. Other factors that affect AMP MOA include various environmental conditions, such as temperature, which may influence AMP secondary structure and membrane fluidity of the bacterial, leading to different interactions [73].

Quartz crystal microbalance with dissipation monitoring (QCM-D) is a powerful tool that has been used to study AMP interactions with SLBs [15, 32, 56, 74]. QCM-D is an acoustic technique where frequency (Δf) and dissipation (ΔD) changes on a quartz sensor surface can be directly related to real-time mass changes (decreasing Δf signifies an increase in mass) and energy dampening (increasing ΔD associated with a less rigid film) [74]. These changes can be monitored over multiple overtones, or harmonics, which can be used to differentiate between processes happening closer to or further away from the sensor surface [15, 16, 32, 56, 62, 74]. Higher overtones are associated with processes closer to the senor surfaces and lower overtones are associated with processes further from the sensor surface [15, 62]. Overtone analysis is powerful in determining the MOA of different AMPs [16, 33, 62, 74]. For example, Wang *et al.* [16] studied Chrysophsin-3 interactions with PC bilayers to examine the cytotoxicity of the peptide using overtone analysis. Lozeau *et al.* [56] examined the effect of different collagen binding domains on the MOA of modified versions of the antimicrobial peptide LL-37 using SLBs. These studies demonstrate the power of using the QCM-D to study the MOA of AMPs using SLBs [15, 56, 62].

Peptide concentration is an import parameter for activity and MOA and often AMPs have a low therapeutic ratio, cytotoxic concentration divided by the minimum inhibitory concentration [8, 15, 16, 56, 75-81]. In the mechanism of pore formation, AMPs concentration is a critical variable [2, 14-16, 56, 80, 81]. At lower concentrations of AMPs, less than 1 uM, pore formation is not guaranteed, as demonstrated by Wang *et al.* [16] and Lozeau *et al.* [56], who showed that the primary peptide interaction with SLBs was adsorption up to a critical concentration. A concentration that is too high is also not appropriate, since chyrsopsin-1 is hemolytic at approximately 10uM [14]. We opted to use 5 uM C-CHY1 based on the results of prior studies with similar peptides [2], as this concentration is within the pore-forming range [2, 14]. We used the QCM-D to form Gram-positive and Gram-negative SLBs incorporating LTA and LPS, respectively, and then examined the influence of temperature and composition on the MOA of C-CHY1 at 5µM concentration. We hypothesized temperature and lipid bilayer composition would have an effect on AMP interaction with the model SLBs. Temperature affects membrane fluidity, which changes the energetic requirements for insertion of C-CHY1 into the membrane [54, 57, 83-85]. At 37^{o}C, due to membrane fluidity, C-CHY1 insertion requires less energy and we expected to observe pore formation. For LPS and LTA we expected larger mass changes due to additional peptide association with these molecules [17, 46, 47, 50, 51, 63, 69, 72]. We also hypothesized that the type of pore formation may be different between SLBs with and without LPS and LTA due to their individual effects on membrane structure and charge [17, 46, 47, 50, 51, 63, 66, 67, 72].

5.2 Materials and methods

5.2.1 Lipids, Lipid Ratios and Peptide Solutions

Lyophilized 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine 16:0-18-1, MW 770.989 g/mol (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine 16:0-18:1, MW 717.996 g/mol (POPE) (Avanti Polar Lipids, city, state), were reconstituted in 100% chloroform. Lyophilized 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) 16:0-18:1, MW 760.076 g/mol (POPG) (Avanti Polar Lipids),

was reconstituted in 97% chloroform 3% methanol v/v solution. Stock concentrations ranged from 20-100mg/mL and were stored under N_2 in -20°C.

The lipid bilayers formed in this study are listed in **Table 1**. The molar ratio for POPC:POPG and POPE:POPG is 4:1, which is a mass ratio of 3.9434:1 (POPC:POPG) and 3.7251:1 (POPE:POPG). The MW of LPS and LTA are both unknown; thus, 10% w/w concentration was used in POPC:POPG LTA and POPE:POPG LPS solutions, relative to the total dried lipid mass. When naming, POPC, POPG and POPE were shortened to PC, PG and PE, respectively.

Table 2: Supported Lipid Bilayer Compositions.

Bilayer Name	**POPC molar ratio[a] (relative to POPG)**	**POPE molar ratio (relative to POPG)**	**LTA (% mass)**	**LPS (% mass)**
PC:PG	**4**	**-**	**-**	**-**
PC:PG LTA	**4**	**-**	**10[b]**	**-**
PE:PG	**-**	**4**	**-**	**-**
PE:PG LPS	**-**	**4**	**-**	**10[b]**

[a]*Molar ratio is 4:1 for all bilayers*

[b]*percent of total mass dried SLB components*

Cysteine modified Chrysophsin-1 (sequence: CFFGWLIKGAIHAGKAIHGLIHRRRH, MW 2994.62 g/mol) was synthesized by Bachem (city, state). The peptide was received as a lyophilized trifluoroacetate salt at greater than 90% purity confirmed by high performance liquid chromatography. Solutions of 5 g/L C-CHY1 were made in PBS (pH 7.2) supplemented with 5 mM ethylenediaminetetraacetic acid as a chelating agent, and stored at -20ºC. C-CHY1 was diluted in MOPS + (described below) at a concentration of 5uM.

5.2.2 Other Reagents/Buffers

All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless specified. Both LPS (*Escherichia coli* O55:B5, MW unknown) and LTA (*Staphylococcus aureus*, MW unknown) in sterile deionized water at a stock concentration of 5 mg/mL and were stored at 4°C. MOPS (3-(N-morpholino)propanesulfonic acid) (MOPS – buffer) was made using 200mL of DI H_2O, 0.419g MOPS, and 1.753g NaCl. The initial pH should be approximately 4.4. NaOH was used to adjust the solution adjusted to pH 7.00 ± 0.05 and filter-sterilized. MOPS +, used when forming SLBs, was made by supplementing filter-sterilized MOPS with 100 mM $CaCl_2$ in order to achieve a final $CaCl_2$ concentration of 2mM.

2.3 Small Unilamellar Vesicle Preparation

After mixing individual lipids, 12 mg (total lipid) were dried with N_2 and desiccated under vacuum overnight. Dried lipids were reconstituted to 2 mg/mL and vortexed thoroughly. To make small unilamellar lipid vesicles, crucial for forming SLBs, the lipid solutions were then subject to five freeze-thaw cycles according to methods described elsewhere [15, 16, 80]. Lipids were then sonicated (Model 150T, ThermoFisher Scientific, Waltham MA) for 60 minutes with a 30% duty (3 seconds on 7seconds off) with a 60% amplitude and then centrifuged for 10 min at 10,000 RPM. The supernatant with vesicles was collected for QCM-D experimentation.

Immediately before flowing lipids through the QCM-D (*section 2.4*), vesicle supernatants were diluted with MOPS(+) to a final liquid concentration of 0.5 mg/mL.

5.2.4 QCM-D Supported Lipid Bilayer Formation

QCM-D (Q-Sense E4 Biolin Scientific, Sweden) was used to form and monitor, in real-time, the SLBs in **Table 1** over silicon dioxide (SiO_2)-coated quartz sensors. SiO_2 sensors were cleaned and prepared prior to each experiment according to published methods [2]. For each experiment, temperature was adjusted using Q-Soft software (Biolin Scientific) to either 23°C or 37°C, depending on experimental condition. Then, MOPS (-) buffer was flowed at 0.150 mL/min to establish an unchanging baseline of ΔD and Δf for all overtones. After a stable baseline was established, the program was restarted and MOPS was

flowed for 2 min. Lipid vesicle solutions (0.5 mg/mL in MOPS(+) buffer) of the desired composition was flowed at 1.5 mL per chamber, followed by flowing MOPS(+) alone for 10 min to ensure SLB formation and remove any excess lipids or salt. Then, 5 µM C-CHY1 in MOPS was flowed at 1.5mL per chamber. Flow was then stopped for a 1 hour static incubation period to allow for peptide-SLB interaction. The experiment was finished after a final 10 min MOPS rinse. Raw QCM-D data of Δf and ΔD vs. time for all overtones (3^{rd}, 5^{th}, 7^{th}, 9^{th} and 11^{th}) were analyzed using QSense DFind software (Biolin Scientific).

5.2.5 Data analysis

Data were analyzed utilizing three major strategies, overtone analysis, dynamic polar plot analysis, and viscoelastic modeling. Q-Sense DFind software (Biolin Scientific) was used to analyze raw QCM-D data using a SmaftFit model to obtain information about both the SLB initial state, thickness and density, as well as model effect C-CHY1 has on the membrane thickness, density and SLB structure. The SmartFit model uses a Voight-Kelvin viscoelastic model using two initial guesses, a thinner more rigid film and a thicker more viscoelastic film [74]. SmartFit finds solutions for both films that minimizes the χ^2 value (a measure of error). Fit quality is given for each solution, a fit quality of 1 corresponds to a perfect fit, the calculated trace perfectly matches the raw data. The better of the two fits was chosen.

For overtone analysis, bar plots were constructed using overtone number vs. average Δf and ΔD and compared from one temperature to another and one composition to another. The technique was developed by Mechler *et al.* [62] and has been used successfully by other researchers such as Wang *et al.* [16] and Lozeau [80] for other membrane-active AMPs. Mechanistic trends may be drawn using overtone analysis. For example, if Δf is negative and the same magnitude across all overtones, this is reflective of a pore formation MOA; whereas, if a significant negative Δf is observed only at lower (3^{rd}, 5^{th}, etc.) overtones, this may reflect AMP surface adsorption [15, 16, 62, 80].

Plots of ΔD vs. Δf (also known as *polar plots*) were constructed for each condition, to help formulate a dynamic picture of C-CHY1 MOA. The changes in frequency and dissipation data were normalized for each overtone, then the change in dissipation and frequency is plotted as the y and x axis respectively (the

positive x axis is increasingly negative) with respect to time. Movement in the positive x direction means an increase in deposited mass. In the y axis, moving upward means a less rigid film and moving downward means a more rigid film. For example, if the plot moves to the right and up, it means more mass is being added and there is an associated change in the SLB becoming less rigid.

5.3 Results and Discussion

Two types of SLBs (PCPG, PCPG+LTA) representing Gram-positive bacteria and two types of SLBs (PEPG, PEPG+LPS) representing Gram-negative bacteria were formed in QCM-D in order to study the effect of temperature and bilayer composition on the MOA of C-CHY1 (**Table 1**). As expected, the formation of SLBs was evident from large negative Δf on the order of -55 to -120 Hz or more as vesicles adsorbed onto the sensor, followed by a transition where Δf increased and stabilized to ~-26 Hz – 40 Hz and ΔD decreased to a relatively rigid value of ~5E-06, as a planar SLB was formed (Supplemental Figure 5 and Figure 6**)** [15, 16].

3.1. *The addition of LTA into SLBs caused significant peptide surface adsorption.*

C-CHY1 did not cause significant overall changes in Δf and ΔD at 23^{o}C for PCPG SLBs over the full interaction period, with the maximum change in Δf as -2.45 Hz, corresponding to 0.34 ng (Figure 1A) and a ΔD of 0.8 x10^{-6} (Figure 1E), at the 3rd overtone. This is similar to what Wang et al. found for Chrysophsin-3 at a similar concentration (4 μM) on SLBs composed of only PC, with Δf of ~-5 Hz and a ΔD of ~ 3x10^{-6} [15].

When LTA was added to the SLBs, however, overall Δf decreased (Figure 1B) and ΔD increased (Figure 1F) across all overtones, suggesting that changes in the interaction were driven by the presence of LTA (Figure 1B). Across all overtones, Δf decreased five to ten-fold (Figure 1B), suggesting increased C-CHY1 adsorption due to LTA. Examination of differences in frequency and dissipation changes as a function of the overtone number can reveal more information on the mechanism of interaction [15, 16, 32, 62]. Since the 3rd overtone was much more negative relative to all other overtones, this suggests pore

formation with peptide surface adsorption [16, 80]. A similar trend was observed for ΔD, with a relatively higher change at the 3rd overtone (Figure 1F), suggesting that the surface of the SLB was more disordered than areas within the bilayer. The higher ΔD 3rd overtone having more disorder than the other overtone can partially be explained by the fact that AMPs forming the pores can act to stabilize the bilayer [86].

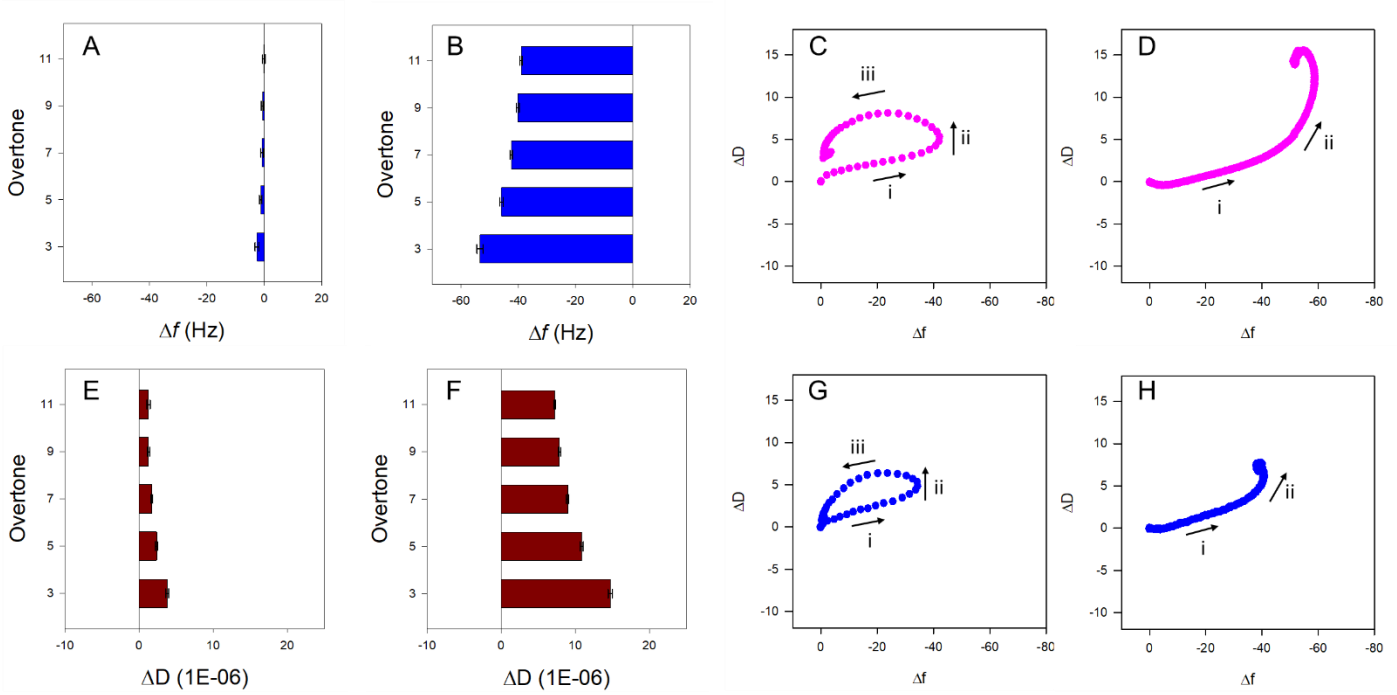


Figure 1 : The overall and dynamic effect of LTA addition to SLBs at 23°C. Plots A, C, E and G are for PCPG bilayers at 23ºC. Plots B, D, F and H are for PCPG+LTA bilayers at 23ºC Overall Δ*f* and ΔD across the 3rd through 11th overtones for **A and E**, PCPG SLBs after C-CHY1 interaction and **B and F**, PCPG+LTA SLBs, after C-CHY1 interaction. ΔD vs. Δ*f* polar plots at the 3rd (C and D) and 11th (G and H) overtones for, PCPG and, PCPG+LTA SLBs respectively. All other overtones followed the same traces and are thus not shown. n>4

The Δ*f* and ΔD QCM-D traces were transformed into ΔD vs. Δ*f* plots to investigate real-time dynamics of the interaction between C-CHY1 and the different bilayers (Figure 1C-1D). Despite demonstrating insignificant changes in Δ*f* and ΔD overall (Figure 1A), it is clear from the polar plots that C-CHY1 interacted dynamically with PCPG SLBs alone, likely driven by electrostatics of the positively

charged residues in C-CHY1 with anionic PG lipids [53, 54, 61, 62, 66, 67]. The shape of the plot is circular with the mechanistic steps, starting at the origin, following a counter-clockwise dynamic (Figure 1C), indicating a 3-step interaction. The first step (indicated by *i*) demonstrated a simultaneous increase in mass and film flexibility (-Δ*f* and +ΔD), indicating the addition of peptide on the surface, until reaching a maximum magnitude Δ*f* of ~-40 Hz, corresponding to a mass of 5.9 ng. The polar plot turned direction at the next step (*ii*) with increased ΔD and no addition of mass. This suggests a purely energetic change of the peptides on the surface, potentially as a threshold for the third step (*iii*), where there is a loss of mass and increased rigidity (+Δ*f* and -ΔD) until reaching the initial state of the bilayer. The mechanisms at step (*i*) and (*iii*) occur relatively quickly compared with step (*ii*), where the spacing between points were closer together. A similarly shaped ΔD vs. Δ*f* plot has been observed for other suspected pore-forming peptides, namely LL37 , which suggests that C-CHY1 forms pores at this concentration [32, 87].

With the incorporation of LTA, the polar plot changes its shape and relative speed (Figure 1D). The shape of the plot indicates a 2-step interaction mechanisms. The first step (*i*) against PCPG+LTA, -Δ*f* and +ΔD, is the same as is demonstrated against PCPG SLBs (Figure 1D vs. Figure 1C, step (*i*)) but is relatively slower as indicated by the close proximity of adjacent data points. This indicates that there is slow addition of peptide on the surface until reaching a maximum Δ*f* of ~-58.8 Hz, corresponding to a mass of 8.2 ng. As with PCPG, the second step (*ii)* represents a turn in direction with no addition of mass but increased ΔD. With the final rinse of the bilayer (represented by **R**) the trace does not return to the original bilayer values of Δ*f* and ΔD but instead remain close to -40 Hz and a high ΔD.

5.3.2 *Increased temperature does not increase interaction dynamics with Gram-positive membranes but alters the overall mechanism of C-CHY1*

Temperature did not have a significant effect on the interaction of C-CHY1 with either Gram-positive bacterial membrane. This can be seen when comparing the mechanism in Figure 1C-D and Figure 2 C-D where the same 3-step and 2-steps mechanisms were obtained for the PCPG and PCPG+LTA bilayers. For the PCPG bilayers, the mechanism proceeds less rapidly and the maximum change in frequency is

slightly lower at 30 Hz, corresponding to 4.34 ng. This suggests that for the Gram-positive membranes the composition, incorporation of LTA, is the most important parameter for MOA of C-CHY1 compared to temperature.

LTA promoted changes in C-CHY1-lipid bilayer interactions (Figure 2A and 2B). Across all overtones, frequency decreased nearly 10 to 20-fold, suggesting significant peptide adsorption and insertion into the bilayer. The 3rd overtone was more negative than the other overtones, meaning additional mass was associated with the top layer, suggesting pore formation with peptide surface adsorption [16, 80]. For ΔD, there was also a relatively higher change at the 3rd overtone, suggesting that surface of the SLB was more disordered. Pore formation caused disruption of the SLB but did not affect membrane stability as much as peptides that adsorb on the surface. [86]. Similar trends were seen at 23^{o}C and 37^{o}C when describing the frequency changes for the LTA SLBs. However, at 37 ^{o}C, the dissipation profile was different for PCPG compared to PCPG at 23^{o}C. Maximum frequency changes of -31.2 Hz (4.33 ng) and -48.6 Hz (6.75 ng) were found for the PCPG and PCPGLTA, respectively.

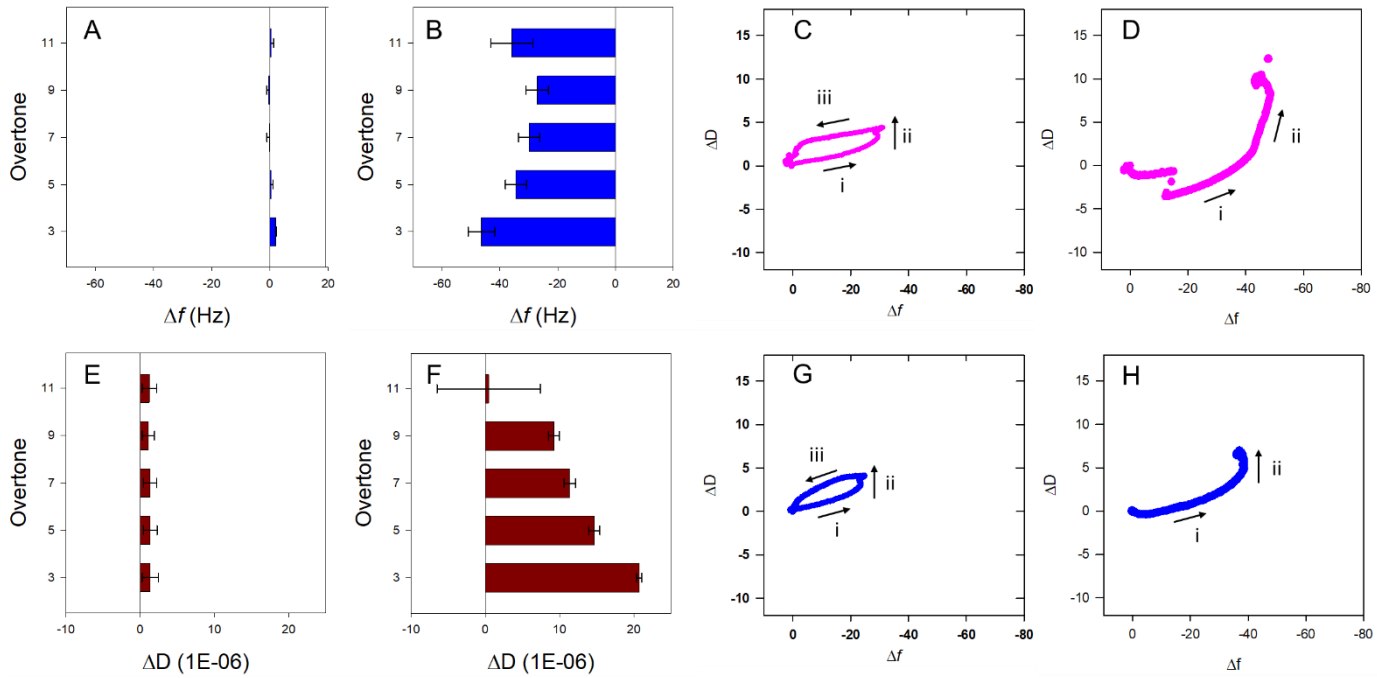


Figure 2: The effect of LTA addition to membranes at physiological temperature, 37°C. Plots A, C, E and G are for PCPG bilayers at 23°C. Plots B, D, F and H are for PCPG+LTA bilayers at 23°C Overall Δ*f* and ΔD across the 3rd through 11th overtones for **A and E**, PCPG SLBs after C-CHY1 interaction and **B and F**, PCPG+LTA SLBs, after C-CHY1 interaction. ΔD vs. Δ*f* polar plots at the 3rd (C and D) and 11th (G and H) overtones for, PCPG and, PCPG+LTA SLBs respectively. All other overtones followed the same traces and are thus not shown. n>4

3.3*. The addition of LPS did not significantly alter the interactions of C-CHY1 with PE/PG SLBs.*

At all overtones (3rd and 11th shown, Figure 3C and 3G) the shape of the plot for PEPG is an elongated "s" shape, indicating a 4-step interaction. The first step (indicated by i) demonstrates an increase in mass and film flexibility (negative f and positive D) simultaneously, indicating the addition of peptide on the surface until reaching f at approximately -40 Hz, corresponding to a mass of 5.9ng. The polar plot turns at this step (ii) with no addition of mass and a significant decrease in D suggesting a much more rigid film possibly due to pore formation. This suggests an energetic change of the peptides on the surface, potentially as a threshold for the third step (iii), where there is a further additional of mass (negative f) surface

adsorption and an increase in D (decreased rigidity) ~ -65Hz (9.2ng) (iv) some mass is lost likely due to removal of peptides and lipids in the rinsing step.

PEPG+LPS showed a similar profile at all overtones (3rd and 11th shown, Figure 3D and 3H) the shape of the plot for is an elongated s shape, indicating a 4-step interaction. The first step (indicated by i) demonstrates an increase in mass and film flexibility (negative f and positive D) simultaneously, indicating the addition of peptide on the surface until reaching f at approximately -40 Hz, corresponding to a mass of 5.9 ng. The polar plot turns at this step (ii) with no addition of mass and a decrease in D suggesting a more rigid film possibly due to pore formation however it is not as significant as with the PEPG membrane alone. This suggests an energetic change of the peptides on the surface and shows that the addition of the LPS makes the membrane easier to rearrange. In the third step (iii), where there is a further additional of mass (negative f) surface adsorption and an increase in D (decreased rigidity) ~ -86 Hz (12 ng) (iv) some mass is lost likely due to removal of peptides and lipids in the rinsing step.

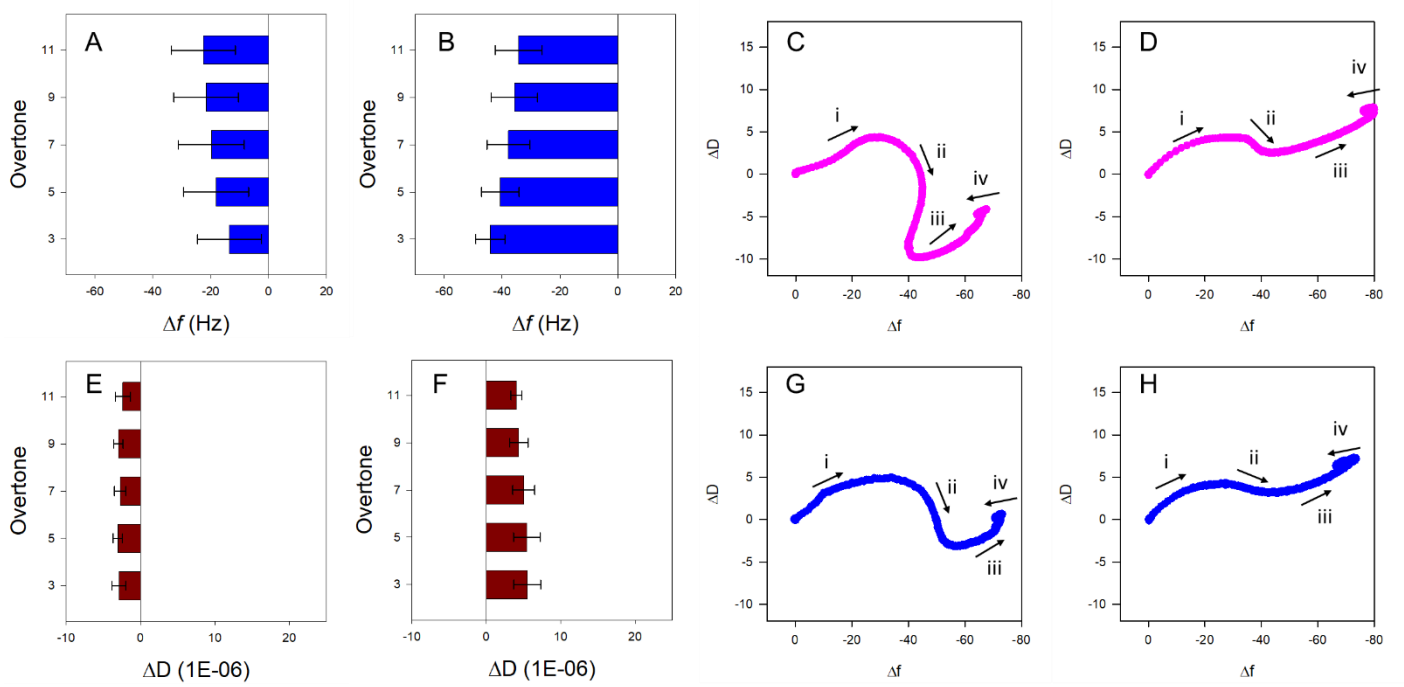


Figure 3: The effect of LPS addition to membranes at 23°C. Plots A, C, E and G are for PEPG bilayers at 23°C. Plots B, D, F and H are for PEPG+LPS bilayers at 23°C Overall Δf and ΔD across the 3rd through

11th overtones for A and E, PCPG SLBs after C-CHY1 interaction and B and F, PEPG+LPS SLBs, after C-CHY1 interaction. ΔD vs. Δf polar plots at the 3rd (C and D) and 11th (G and H) overtones for, PEPG and, PEPG+LPS SLBs respectively. All other overtones followed the same traces and are thus not shown. n>4

5.3.4 Increased temperature did significantly alter the mechanism of C-CHY1 with Gram-negative model SLBs

Temperature did have a significant effect on the interaction of C-CHY1 with Gram-negative membranes. This can be seen when comparing the mechanism in Figure 3C and Figure 4C where the same 4 step mechanism was obtained for the PEPG bilayers as well as for the PEPG+LPS bilayers however there are a few major differences. For step (ii) the energetic requirement for peptide insertion is much lower as seen in Figure 4C compared to Figure 3C with a much lower slope. This is due to the fact that the glass transition temperature of PE is 25ºC (Avanti Polar Lipids). Therefore the PEPG membrane at 23ºC is less fluid but at 37ºC the membrane is more fluid thus it is easier for the C-CHY1 to insert into the membrane, maximum change in *f* ~55 (7.7ng) compared to 9.2 ng. For PEPG+LPS the maximum change in frequency was ~48 (6.6 ng). Additionally the is more mass loss in step (iv) which may suggest that with a more fluid membrane lipids are more easily removed (Figure 3C and 4C). Based on Figure 4A compared to Figure 3A there appears to be a slight difference in the change frequency and dissipation profiles as well. At 37ºC with PEPG+LPS SLBs the MOA appears to be pore formation only, while at 23ºC, there appears to be slightly less pore formation but more peptide adsorption on the surface, likely due to a more rigid film. Overall, the MOA against Gram-negative SLBs appears to be pore formation with temperature being the most important variable in C-CHY1 interaction.

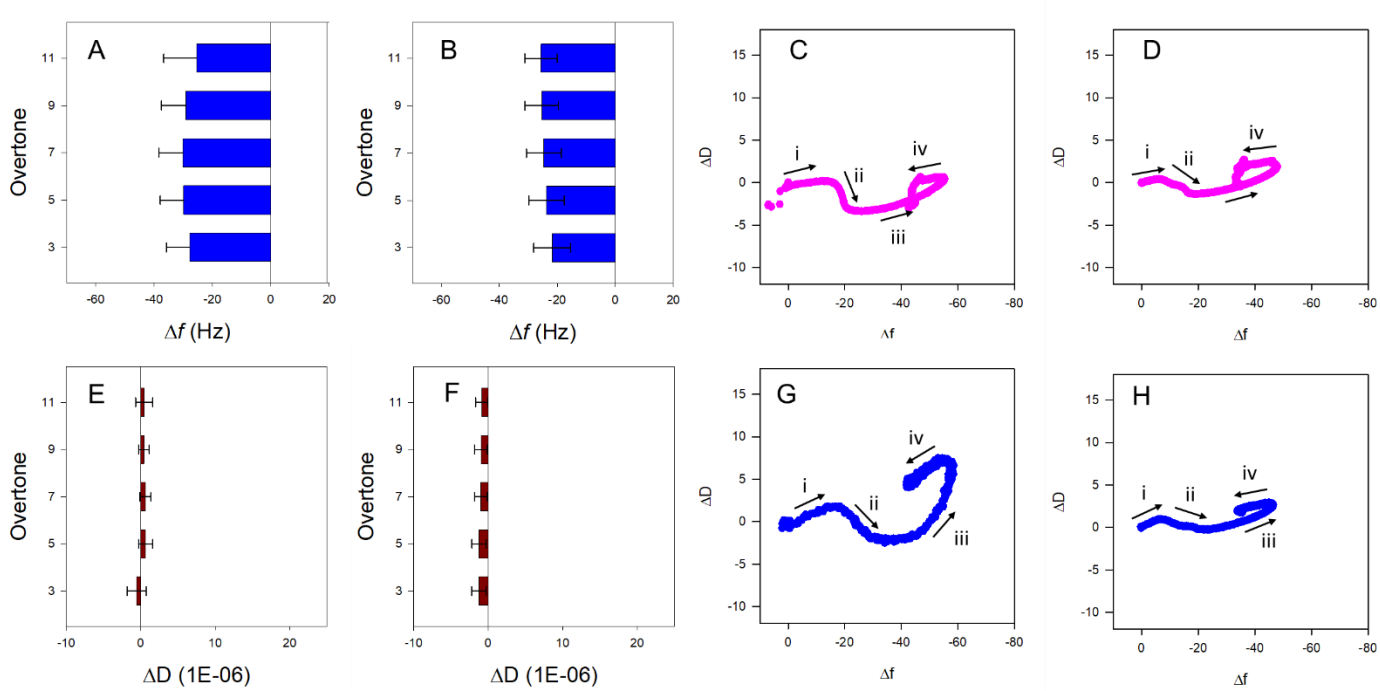


Figure 4: The effect of LPS addition to membranes at 37°C. Plots A, C, E and G are for PEPG bilayers at 23°C. Plots B, D, F and H are for PEPG+LPS bilayers at 23°C Overall Δf and ΔD across the 3rd through 11th overtones for A and E, PCPG SLBs after C-CHY1 interaction and B and F, PEPG+LPS SLBs, after C-CHY1 interaction. ΔD vs. Δf polar plots at the 3rd (C and D) and 11th (G and H) overtones for, PEPG and, PEPG+LPS SLBs respectively. All other overtones followed the same traces and are thus not shown. n>4

5.3.5. *Overall changes in bilayer thickness reveal mechanistic differences between Gram-positive and Gram-negative bacterial model membranes.*

For the PEPG and PEPG+LPS membranes, the major factor affecting MOA was temperature. This differs in the comparison between PCPG and PCPG+LTA where the SLB composition was the most important factor affecting the MOA. However, there was some slight influence in temperature on the kinetics of interaction as for Gram-positive SLBs, as seen in the polar plots (Figure 1C-D and Figure 2C-D). Additionally, in the Gram-negative membranes there was a higher kinetic barrier to peptide insertion compared to Gram-positive membranes step (ii) in the kinetic models for MOA for all membranes. For Gram-positive membranes, differences can be seen in changes in the SLB thickness (Figure 5 A and C). For Gram-positive SLBs with LTA the membrane thickens with C-CHY1 interaction. This suggests that C-

CHY1 interacts with the LTA in a way that either causes swelling of the bulk membrane or saturation of the LTA causing it to fully extend. When combined with the polar plot, frequency and dissipation data, this suggests that the peptide is not only forming pores but saturating the LTA causing it to extend fully; rather than causing a thickening of PCPG and anchor portion of the LTA. With the Gram-negative membranes the trends in changing thickness were the same for membranes with and without LPS with the difference being the change in starting thickness due to the LPS (Figure 5B and D). When the C-CHY1 interacts with the Gram-negative bilayers and forms pores, the membrane thins slightly (Figure 5B and D). For PEPG bilayers, the membrane thins to the thickness of a C-CHY1 dimer (3.5 nm in length) [19]. This suggests the formation of peptide dimers which is likely due to the cysteine modification [16, 88]. Combined with the polar plots, frequency and dissipation data, this suggests that for Gram-negative SLBs, the MOA is pore formation via C-CHY1 dimers and that there is no preferential interaction of C-CHY1 with the LPS over the bulk PEPG.

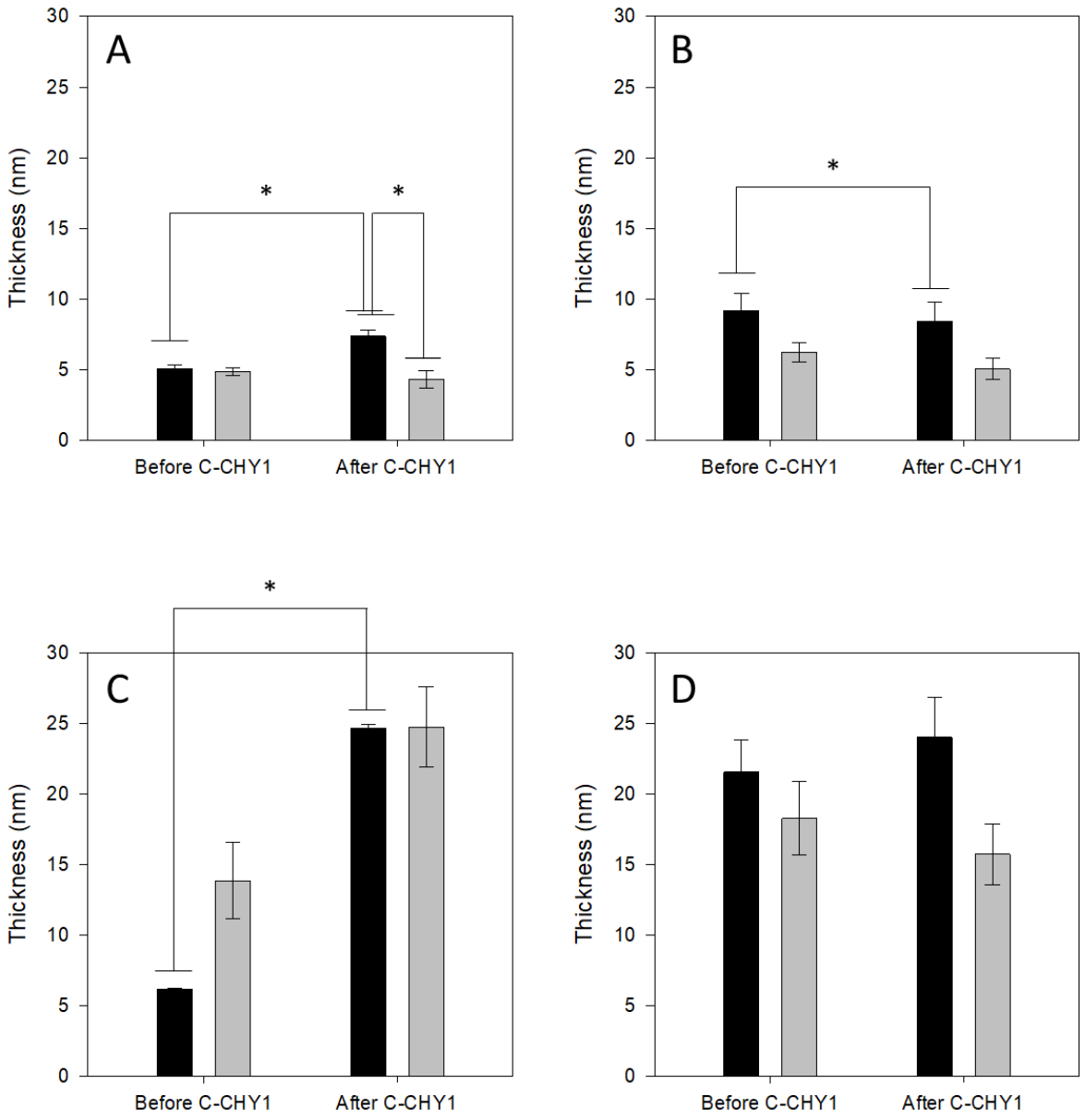


Figure 5 : The overall bilayer thickness as a result of the addition of molecules to mimic Gram-positive and Gram-negative bacterial membranes, LTA and LPS. A) The thickness of PCPG bilayers before peptide interaction and after peptide interaction. B) The thickness of PEPG bilayers before peptide interaction and after peptide interaction. C) The thickness of PCPG+LTA bilayers before peptide interaction and after peptide interaction. D) The thickness of PEPG+LPS bilayers before peptide interaction and after peptide interaction. Statistical significance of $p<0.05$ was determined using a One Way Anova with a post hoc Tukey Test. $n>4$

5.3.6 *Predictions of maximum f to theoretical values of peptide mechanisms suggests C-CHY1 is a pore former against both Gram-positive and Gram-negative bacterial membranes.*

Theoretical calculations of peptide interactions with the model bacterial membranes were calculated for various mechanisms of action, adsorption, insertion as a monomer, insertion as a cluster, and pore formation (8 and 20 peptides). The calculations are based on the calculations performed in Wang *et al.* [16]. Modifications needed to be made to the equations as the Wang *et al.* [16] model is for one component bilayer systems. The key modifications are as follows. For the molecular mass of the lipid M_L the molecular mass of the lipid becomes $M_{L,Average}$ where the molar ratio of the lipids is used to come up with an average "unit" molecular weight. For the LPS and LTA components molecular weight values were taken from the literature and a molar ratio was calculated based on the mass added in each bilayer composition [61, 89]. For the lipid area a_L was adjusted to $a_{L,Average}$ with a weighted average based on molar ratio.

Peptide dimerization was considered for the same conditions as well due to the cysteine residue which can promote dimerization [16, 88]. It is important to note that the calculations are based on 10% or maximum frequency changes for each of the mechanisms. Additionally the average molecular weight of the LTA and LPS in unknown which effects the accuracy of the theoretical results. For *E.coli* LPS the MW is based on the molecular weights reported using the same strain O55:B5 in Peterson *et al.* [89]. For the *S. aureus* LTA the molecular weight was taken from an average reported by Malanovic *et al.* [61] and may not be accurate for the LTA used in this study. This give a range a frequency changes and thus needs to be combined with other data collected in order to make sense, Table 2. Combining the theoretical calculations with the polar plots, overall frequency and overall dissipation changes we can predict the MOA of the peptide against each of the SLBs. Based on the frequency changes, >-10Hz, for PCPC at both 23^{o}C and 37^{o}C it suggests pore formation as dimers with 20 peptides when combined with the mechanistic steps in the polar plot, Figures 1A and 2A, and changes in membrane thickness, Figure 5A. With PCPG+LTA there was a much more significant decrease in frequency, Figures 1B and 2B, which suggest the formation of pores

with 8 peptides dimers per pore combined with surface adsorption due to the higher change in frequency in Figures 1A and 2A. This is in agreement with prior results, which have shown that AMPs are electrostatically attracted to LTA [46, 61, 71, 72]. For the Gram-negative membranes PEPG and PEPG+LPS, at 23^{o}C and 37^{o}C, the changes in frequency, Figures 3 and 4, suggest pore formation via 8 peptides dimers per pore as well as surface adsorption of C-CHY1 as observed in the higher change in frequency in the 3rd overtone [15, 16, 56]. It is important to note that in this case that the number of peptides per pore is speculative and needs to validated using other techniques such as AFM or X-ray scattering [81].

Table 2: Mechanistic predictions of fmax for C-CHY1 against SLBs.

Lipid Composition	Expected ΔF (Hz) Peptide Monomer							
	Insertion as a monomer or cluster ΔF 10%	Insertion as a monomer or cluster ΔF Max	Pore formation with 8 peptides/pore ΔF 10%	Pore formation with 8 peptides/pore ΔF Max	Pore formation with 20 peptides/pore ΔF 10%	Pore formation with 20 peptides/pore ΔF Max	Adsorption ΔF 10%	Adsorption ΔF Max
PEPG	-3.97	-39.69	0.09	0.87	1.24	12.38	-15.38	-153.83
PEPG LPS	22.24	222.44	26.30	263.01	27.45	274.52	-15.38	-153.83
PCPG	-3.44	-34.42	0.61	6.15	1.77	17.66	-15.38	-153.83
PCPGLTA	5.08	50.83	9.14	91.39	10.29	102.90	-15.38	-153.83

Lipid Composition	Expected ΔF (Hz) Peptide Dimer							
	Insertion as a monomer or cluster ΔF 10%	Insertion as a monomer or cluster ΔF Max	Pore formation with 8 peptides/pore ΔF 10%	Pore formation with 8 peptides/pore ΔF Max	Pore formation with 20 peptides/pore ΔF 10%	Pore formation with 20 peptides/pore ΔF Max	Adsorption ΔF 10%	Adsorption ΔF Max
PEPG	-19.35	-193.52	-11.24	-112.39	-8.94	-89.37	-30.77	-307.66
PEPG LPS	6.86	68.61	14.97	149.75	17.28	172.77	-30.77	-307.66
PCPG	-18.82	-188.25	-10.71	-107.11	-8.41	-84.09	-30.77	-307.66
PCPGLTA	-10.30	-103.00	-2.19	-21.87	0.12	1.15	-30.77	-307.66

5.3.7. *Proposed mechanism of C-CHY1 against bacterial membranes and implications for C-CHY1 clinical applications.*

The proposed MOA of C-CHY1 against both Gram-positive and Gram-negative bacteria is via pore formation however there are differences in the mechanistic steps between the membranes (Figures 6 and 7). Pore formation as the MOA was not surprising as the mechanism of action for Chrysophsin-3 is known to be pore formation at these concentrations [15]. The difference in mechanisms between membranes is surprising as Chrysophsin-1 has a similar MIC for both Gram-positive and Gram-negative bacteria [2, 14, 90, 91].

For the Gram-positive membranes the following mechanisms are proposed. With PCPG SLBs, at 23^{o}C and 37^{o}C, there is initial adsorption onto the SLB due to electrostatic interactions of the positively charged C-CHY1 and the overall negatively charged SLB, Figure 6 step (i). At a threshold concentration the peptide begins to insert into the SLB, step (ii), followed by insertion as 20 peptide dimers units (40 total

peptides) per pore, step (iii). For PCPG+LTA membranes the mechanism is similar however in steps (i) and (iii) where saturation of C-CHY1, electrostatically attracted to the LTA molecules occurs.

For the Gram-negative membranes the following mechanisms are proposed. With PEPG at 23^{o}C there is initial adsorption onto the SLB due to electrostatic interactions of the positively charged C-CHY1 and the overall negatively charged SLB, Figure 7 step (i). After a threshold concentration of C-CHY1 there is a near instantaneous formation of pores as seen in, Figure 3D and Figure 7 step (ii). This is due to the fact that the PEPG bilayer at this temperature is more crystalline than fluid due to the glass transition temperature of PE which is 25 ^{o}C which makes up 80% of the molecules in the SLB. This is followed by additional adsorption of C-CHY1 onto the surface of the SLB due to electrostatic interactions, Figure 7 step (iii). For this condition the pores are formed with peptide dimers with 8 peptide dimer units per pore (16 peptides total). With PEPG at 37^{o}C there is initial adsorption onto the SLB due to electrostatic interactions of the positively charged C-CHY1 and the overall negatively charged SLB, Figure 7 step (i). At a threshold concentration the peptide begins to insert into the SLB, step (ii), followed by insertion with 8 peptide dimer units per pore (16 peptides total), step (iii). This is followed by some mass loss of peptide-lipid aggregates step (iv).

With PEPG+LPS at 23^{o}C and 37^{o}C there is initial adsorption onto the SLB due to electrostatic interactions of the positively charged C-CHY1 and the overall negatively charged SLB, Figure 7 step (i). At a threshold concentration the peptide begins to insert into the SLB, step (ii), followed by insertion with 8 peptide dimer units per pore (16 peptides total), step (iii). This is followed by some mass loss of peptide-lipid aggregates step (iv).

It was uncertain how the cysteine modification would affect the MOA of C-CHY1 [90, 91]. For example, Tripathi *et al.* [91] altered the amino acid sequence of Chrysophsin-1 with no significant effect on its activity against Gram-positive bacteria. However, the activity against Gram-negative for most of the analogs was reduced [91]. This may suggest that one must be careful when making amino acid substitutions,

deletions, or additions as the activity against Gram-negative bacteria seems particularly susceptible to these changes [2, 90, 91].

These differences between our Gram-positive and Gram-negative membranes include the number of peptides associated with each pore as well as how the peptides associate with and interact with the membrane. For the Gram-negative PEPGLPS membrane there is not a significant change in thickness however for the Gram-positive PCPGLTA membrane there is a significant thickening of the SLB. Also based on the difference in the polar plots there seems to be a higher energetic barrier for C-CHY1 insertion into the Gram-negative membrane. This suggests that it may be easier for C-CHY1 to form pores in and kill Gram-positive bacteria such as S. aureus. This is in agreement with our findings for both the MIC we previously found as well as with our tethered peptide data see Lozeau *et al.* [2] and Supplementary Figures 4.

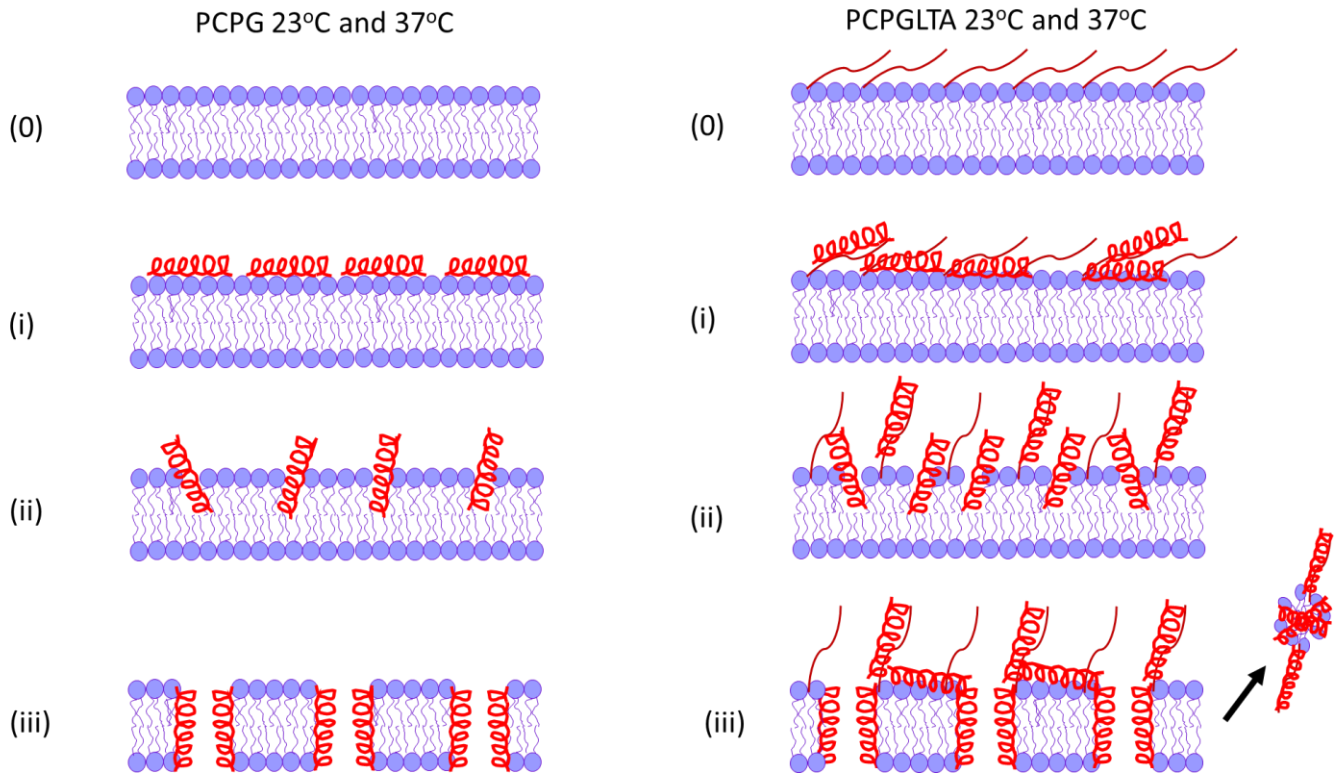


Figure 6: Proposed mechanism of action of C-CHY1 against representative Gram-positive bacterial membranes. A) PCPG membranes at 23^{o}C and 37^{o}C. (i) Initial adsorption of the peptide on the membrane

(ii) Rearrangement of the membrane to allow for peptide insertion (iii) formation of pores in the membrane. B) PCPGLTA membranes at 23^{o}C and 37^{o}C. (i) adsorption of the peptide on the membrane surface and association with the LTA. (ii) Rearrangement of the membrane to allow for peptide insertion as well as continued binding to the LTA (iii) Membrane pores with adsorption of C-CHY1 onto the surface and LTA until saturation with a small amount of lipid removal.

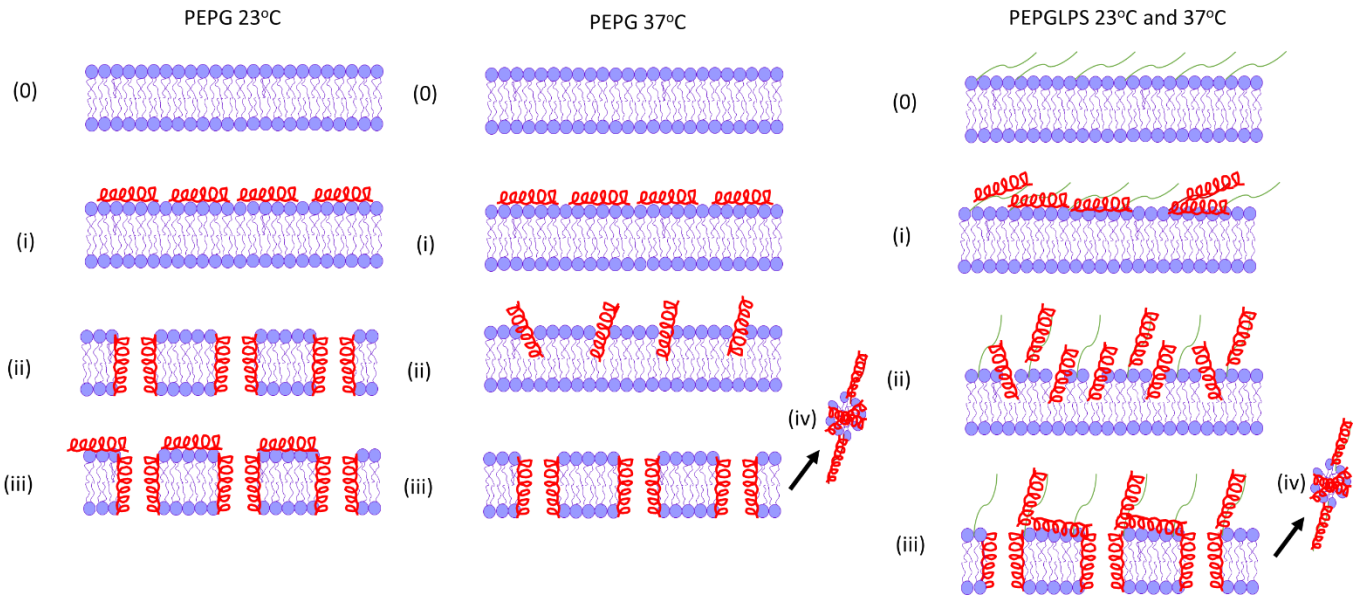


Figure 7: Proposed mechanism of action of C-CHY1 against representative Gram-negative bacterial membranes. A) PEPG membranes at 23^{o}C. (i) Initial adsorption of the peptide on the membrane until a critical point (ii) Rearrangement of the membrane to allow for peptide insertion and rapid pore formation due to crystallinity/rigidity of the membrane (iii) adsorption of the peptide on the surface. B) PEPG membranes at 37^{o}C. (i) Initial adsorption of the peptide on the membrane until a critical point (ii) Rearrangement of the membrane to allow for peptide insertion (iii) pore formation (iv) mass loss of the membrane as peptide lipid aggregates. C) PEPG+LPS membranes at 23^{o}C and 37^{o}C. (i) adsorption of the peptide on the membrane surface and association with the LPS. (ii) Rearrangement of the membrane to

allow for peptide insertion as well as continued binding to the LPS (iii) Membrane pores with adsorption of C-CHY1 onto the surface and LPS until with a lipid removal as peptide lipid aggregates.

5.4 Summary and conclusion

QCM-D and the use of SLBs is an extremely powerful technique that allows for the study of AMP MOA in a systematic way [15, 16, 32]. The complexity of the SLB as well as testing conditions such as temperature can have a significant effect on the MOA [15, 54, 57]. For C-CHY1 we were able to use SLBs to confirm that cysteine modified Chrysophsin-1 uses a similar pore formation MOA that its unmodified counterpart, CHY1, uses. Additionally we were able to determine that even though the end result was the same for Gram-negative and Gram-positive SLBs, pore formation, the mechanistic steps were different. We were able to suggest that C-CHY1 forms pores more easily in Gram-positive membranes which may inform the best way to clinically apply it. Additionally we demonstrated that the QCM-D can be used to study how various proteins such as LPS and LTA effect the MOA of C-CHY1. For the Gram-negative membranes LPS did not have a significant effect on MOA. For the Gram-positive membranes LTA did have a significant effect on the MOA, with more peptide associating with the bilayer. Temperature did have an effect on the interaction of C-CHY1 with the Gram-negative membrane likely due to the melting temperature of the lipids which effected bilayer fluidity. Our study suggests that C-CHY1 should be used in clinical applications where Gram-positive bacteria are a major concern such as skin infections or used in combination with another AMP or antibiotic for Gram-negative infections [3, 7, 92]. This also suggests that when altering Chrysophsin-1 it is important to note that activity against Gram-negative bacteria is sensitive to amino acid sequence changes [90, 91]. A direction for future research may be to look into what key structures and amino acids are important for Chrysophsin-1 activity against Gram-negative bacteria. This will allow for more rational design of the peptide in the future allowing for the maintenance of its naturally broad spectrum activity. Use of SLBs for this application can be used to screen other AMPs and inform clinical

targets. This technique can be expanded to examine the effect of other surface proteins, however complexity should be balanced with the ability to interpret the data.

I would like to thank Dr. Ramanathan Nagarajan for his assistance in interpreting the Q-CMD data.

References

1. O'Neill, J., *Infection prevention, control and surveillance: limiting the development and spread of drug resistance. The review on antimicrobial resistance*. 2016.
2. Lozeau, L.D., T.E. Alexander, and T.A. Camesano, *Proposed mechanisms of tethered antimicrobial peptide chrysophsin-1 as a function of tether length using QCM-D.* The Journal of Physical Chemistry B, 2015. **119**(41): p. 13142-13151.
3. Marr, A.K., W.J. Gooderham, and R.E. Hancock, *Antibacterial peptides for therapeutic use: obstacles and realistic outlook.* Current opinion in pharmacology, 2006. **6**(5): p. 468-472.
4. Hancock, R.E. and R.J.T.i.b. Lehrer, *Cationic peptides: a new source of antibiotics.* 1998. **16**(2): p. 82-88.
5. Hancock, R.E.J.T.L.i.d., *Cationic peptides: effectors in innate immunity and novel antimicrobials.* 2001. **1**(3): p. 156-164.
6. Jenssen, H., P. Hamill, and R.E.J.C.m.r. Hancock, *Peptide antimicrobial agents.* 2006. **19**(3): p. 491-511.
7. Hancock, R.E. and H.-G. Sahl, *Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies.* Nature biotechnology, 2006. **24**(12): p. 1551.
8. Deslouches, B., et al., *Rational design of engineered cationic antimicrobial peptides consisting exclusively of arginine and tryptophan, and their activity against multidrug-resistant pathogens.* Antimicrobial agents and chemotherapy, 2013. **57**(6): p. 2511-2521.
9. Dale, B.A. and L.P. Fredericks, *Antimicrobial peptides in the oral environment: expression and function in health and disease.* Current issues in molecular biology, 2005. **7**(2): p. 119.

10. Hancock, R.E.W., *The bacterial outer membrane as a drug barrier.* Trends in Microbiology, 1997. **5**(1): p. 37-42.

11. Smart, M., et al., *Opposing effects of cationic antimicrobial peptides and divalent cations on bacterial lipopolysaccharides.* Physical Review E, 2017. **96**(4): p. 042405.

12. Reddy, K., R. Yedery, and C. Aranha, *Antimicrobial peptides: premises and promises.* International journal of antimicrobial agents, 2004. **24**(6): p. 536-547.

13. Brogden, K.A., *Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?* Nature reviews microbiology, 2005. **3**(3): p. 238.

14. Iijima, N., et al., *Purification and characterization of three isoforms of chrysophsin, a novel antimicrobial peptide in the gills of the red sea bream, Chrysophrys major.* European Journal of Biochemistry, 2003. **270**(4): p. 675-686.

15. Wang, K.F., et al., *Characterization of supported lipid bilayer disruption by chrysophsin-3 using QCM-D.* The Journal of Physical Chemistry B, 2011. **115**(51): p. 15228-15235.

16. Wang, K.F., R. Nagarajan, and T.A. Camesano, *Differentiating antimicrobial peptides interacting with lipid bilayer: molecular signatures derived from quartz crystal microbalance with dissipation monitoring.* Biophysical chemistry, 2015. **196**: p. 53-67.

17. Michel, J.-P., et al., *Disruption of Asymmetric Lipid Bilayer Models Mimicking the Outer Membrane of Gram-Negative Bacteria by an Active Plasticin.* Langmuir, 2017. **33**(41): p. 11028-11039.

18. Batoni, G., et al., *Use of antimicrobial peptides against microbial biofilms: advantages and limits.* Current medicinal chemistry, 2011. **18**(2): p. 256-279.

19. Ivanov, I.E., et al., *Creating Antibacterial Surfaces with the Peptide Chrysophsin-1.* ACS Applied Materials & Interfaces, 2012. **4**(11): p. 5891-5897.

20. Chung, K.K., et al., *Impact of engineered surface microtopography on biofilm formation of Staphylococcus aureus.* Biointerphases, 2007. **2**(2): p. 89-94.

21. Tobin, E.J., *Recent coating developments for combination devices in orthopedic and dental applications: A literature review.* Advanced Drug Delivery Reviews, 2017. **112**: p. 88-100.

22. Sullivan, F., *U. S. Urinary Incontinence Devices*, F. Sullivan, Editor. 2011.

23. Yu, K., et al., *Anti-adhesive antimicrobial peptide coating prevents catheter associated infection in a mouse urinary infection model.* Biomaterials, 2017. **116**: p. 69-81.

24. Pickard, R., et al., *Antimicrobial catheters for reduction of symptomatic urinary tract infection in adults requiring short-term catheterisation in hospital: a multicentre randomised controlled trial.* The lancet, 2012. **380**(9857): p. 1927-1935.

25. Bonfill, X., et al., *Efficacy and safety of urinary catheters with silver alloy coating in patients with spinal cord injury: a multicentric pragmatic randomized controlled trial. The ESCALE trial.* The Spine Journal, 2017. **17**(11): p. 1650-1657.

26. Kennedy, E.H., M.T. Greene, and S.J.J.o.h.m. Saint, *Estimating hospital costs of catheter-associated urinary tract infection.* 2013. **8**(9): p. 519-522.

27. Control, C.f.D. and Prevention, *Urinary tract infection (catheter-associated urinary tract infection [CAUTI] and non-catheter-associated urinary tract infection [UTI]) and other urinary system infection [USI]) events.* Centers for Disease Control and Prevention, Atlanta, GA: http://www. cdc. gov/nhsn/PDFs/pscManual/7pscCAUTIcurrent. pdf, 2015.

28. Kazemzadeh-Narbat, M., et al., *Antimicrobial peptides on calcium phosphate-coated titanium for the prevention of implant-associated infections.* Biomaterials, 2010. **31**(36): p. 9519-9526.

29. Kostakioti, M., M. Hadjifrangiskou, and S.J. Hultgren, *Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era.* Cold Spring Harbor perspectives in medicine, 2013. **3**(4): p. a010306-a010306.

30. Gao, G., et al., *Biomembrane Interactions Reveal the Mechanism of Action of Surface-Immobilized Host Defense IDR-1010 Peptide.* Chemistry & Biology, 2012. **19**(2): p. 199-209.

31. Otto, K., H. Elwing, and M. Hermansson, *Effect of Ionic Strength on Initial Interactions ofEscherichia coli with Surfaces, Studied On-Line by a Novel Quartz Crystal Microbalance Technique.* Journal of Bacteriology, 1999. **181**(17): p. 5210-5218.

32. Lozeau, L.D., et al., *Concentration-Dependent, Membrane-Selective Activity of Human LL37 Peptides Modified with Collagen Binding Domain Sequences.* Biomacromolecules, 2018.

33. Lozeau, L.D., et al., *A QCM-D study of the concentration-and time-dependent interactions of human LL37 with model mammalian lipid bilayers.* 2018. **167**: p. 229-238.

34. Le, C.-F., C.-M. Fang, and S.D. Sekaran, *Intracellular Targeting Mechanisms by Antimicrobial Peptides.* Antimicrobial Agents and Chemotherapy, 2017. **61**(4): p. e02340-16.

35. Monzillo, V., et al., *Chlorhexidine-silver sulfadiazine-impregnated central venous catheters: in vitro antibacterial activity and impact on bacterial adhesion.* 2012. **35**(2): p. 175.

36. Bagheri, M., M. Beyermann, and M. Dathe, *Immobilization reduces the activity of surface-bound cationic antimicrobial peptides with no influence upon the activity spectrum.* Antimicrobial agents and chemotherapy, 2009. **53**(3): p. 1132-1141.

37. Onaizi, S.A. and S.S. Leong, *Tethering antimicrobial peptides: current status and potential challenges.* Biotechnology advances, 2011. **29**(1): p. 67-74.

38. Costa, F., et al., *Covalent immobilization of antimicrobial peptides (AMPs) onto biomaterial surfaces.* Acta biomaterialia, 2011. **7**(4): p. 1431-1440.

39. Haynie, S.L., G.A. Crum, and B.A. Doele, *Antimicrobial activities of amphiphilic peptides covalently bonded to a water-insoluble resin.* Antimicrobial Agents and Chemotherapy, 1995. **39**(2): p. 301-307.

40. Hilpert, K., et al., *Screening and characterization of surface-tethered cationic peptides for antimicrobial activity.* Chemistry & biology, 2009. **16**(1): p. 58-69.

41. Lozeau, L.D., et al., *Collagen tethering of synthetic human antimicrobial peptides cathelicidin LL37 and its effects on antimicrobial activity and cytotoxicity.* 2017. **52**: p. 9-20.

42. Li, Y., et al., *Effects of peptide immobilization sites on the structure and activity of surface-tethered antimicrobial peptides.* The Journal of Physical Chemistry C, 2015. **119**(13): p. 7146-7155.

43. Kügler, R., O. Bouloussa, and F. Rondelez, *Evidence of a charge-density threshold for optimum efficiency of biocidal cationic surfaces.* Microbiology, 2005. **151**(5): p. 1341-1348.

44. Lienkamp, K., et al., *Antimicrobial Polymers Prepared by Ring-Opening Metathesis Polymerization: Manipulating Antimicrobial Properties by Organic Counterion and Charge Density Variation.* Chemistry–A European Journal, 2009. **15**(43): p. 11715-11722.

45. Imura, Y., M. Nishida, and K. Matsuzaki, *Action mechanism of PEGylated magainin 2 analogue peptide.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2007. **1768**(10): p. 2578-2585.

46. Nawrocki, L.K., K.E. Crispell, and M.S. McBride, *Antimicrobial Peptide Resistance Mechanisms of Gram-Positive Bacteria.* Antibiotics, 2014. **3**(4).

47. Nizet, V., *Antimicrobial peptide resistance mechanisms of human bacterial pathogens.* Current issues in molecular biology, 2006. **8**(1): p. 11.

48. Wimley, W.C. and K. Hristova, *Antimicrobial Peptides: Successes, Challenges and Unanswered Questions.* The Journal of Membrane Biology, 2011. **239**(1): p. 27-34.

49. Torcato, I.M., et al., *Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2013. **1828**(3): p. 944-955.

50. Han, H.M., R. Gopal, and Y. Park, *Design and membrane-disruption mechanism of charge-enriched AMPs exhibiting cell selectivity, high-salt resistance, and anti-biofilm properties.* Amino Acids, 2016. **48**(2): p. 505-522.

51. Andersson, D.I., D. Hughes, and J.Z. Kubicek-Sutherland, *Mechanisms and consequences of bacterial resistance to antimicrobial peptides.* Drug Resistance Updates, 2016. **26**: p. 43-57.

52. Papo, N. and Y. Shai, *A molecular mechanism for lipopolysaccharide protection of Gram-negative bacteria from antimicrobial peptides.* Journal of Biological Chemistry, 2005. **280**(11): p. 10378-10387.

53. Richter, R.P. and A.R. Brisson, *Following the formation of supported lipid bilayers on mica: a study combining AFM, QCM-D, and ellipsometry.* Biophysical journal, 2005. **88**(5): p. 3422-3433.

54. Hardy, G.J., R. Nayak, and S. Zauscher, *Model cell membranes: Techniques to form complex biomimetic supported lipid bilayers via vesicle fusion.* Current opinion in colloid & interface science, 2013. **18**(5): p. 448-458.

55. Flynn, K.R., et al., *Real-time quartz crystal microbalance monitoring of free docosahexaenoic acid interactions with supported lipid bilayers.* Langmuir, 2016. **32**(45): p. 11717-11727.

56. Lozeau, L.D., et al., *Concentration-Dependent, Membrane-Selective Activity of Human LL37 Peptides Modified with Collagen Binding Domain Sequences.* Biomacromolecules, 2018. **19**(12): p. 4513-4523.

57. Kamaloo, E., *Supported lipid bilayer interactions with nanoparticles, peptides and polymers.* 2018.

58. Lee, T.-H., D.J. Hirst, and M.-I. Aguilar, *New insights into the molecular mechanisms of biomembrane structural changes and interactions by optical biosensor technology.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2015. **1848**(9): p. 1868-1885.

59. Hussain, Noor F., et al., *Bilayer Asymmetry Influences Integrin Sequestering in Raft-Mimicking Lipid Mixtures.* Biophysical Journal, 2013. **104**(10): p. 2212-2221.

60. Hedegaard, S.F., et al., *Fluorophore labeling of a cell-penetrating peptide significantly alters the mode and degree of biomembrane interaction.* Scientific reports, 2018. **8**(1): p. 6327.

61. Malanovic, N. and K. Lohner, *Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2016. **1858**(5): p. 936-946.

62. Mechler, A., et al., *Specific and selective peptide-membrane interactions revealed using quartz crystal microbalance.* Biophysical Journal, 2007. **93**(11): p. 3907-3916.

63. Ebbensgaard, A., et al., *The Role of Outer Membrane Proteins and Lipopolysaccharides for the Sensitivity of Escherichia coli to Antimicrobial Peptides.* Frontiers in microbiology, 2018. **9**: p. 2153-2153.

64. Brandenburg, K., et al., *Peptides with dual mode of action: Killing bacteria and preventing endotoxin-induced sepsis.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2016. **1858**(5): p. 971-979.

65. Costa, F., et al., *Clinical Application of AMPs*, in *Antimicrobial Peptides: Basics for Clinical Application*, K. Matsuzaki, Editor. 2019, Springer Singapore: Singapore. p. 281-298.

66. Matsuzaki, K., *Control of cell selectivity of antimicrobial peptides.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2009. **1788**(8): p. 1687-1692.

67. Silhavy, T.J., D. Kahne, and S. Walker, *The bacterial cell envelope.* Cold Spring Harbor perspectives in biology, 2010. **2**(5): p. a000414-a000414.

68. Denyer, S.P. and J.Y. Maillard, *Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria.* Journal of Applied Microbiology, 2002. **92**(s1): p. 35S-45S.

69. Beveridge, T.J., *Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles.* Journal of Bacteriology, 1999. **181**(16): p. 4725.

70. Freire, J.M., et al., *Shifting gear in antimicrobial and anticancer peptides biophysical studies: from vesicles to cells.* Journal of peptide Science, 2015. **21**(3): p. 178-185.

71. Scott, M.G., M.R. Gold, and R.E. Hancock, *Interaction of cationic peptides with lipoteichoic acid and gram-positive bacteria.* Infection and immunity, 1999. **67**(12): p. 6445-6453.

72. Brown, S., J.P. Santa Maria, Jr., and S. Walker, *Wall teichoic acids of gram-positive bacteria.* Annual review of microbiology, 2013. **67**: p. 313-336.

73. Rydlo, T., J. Miltz, and A. Mor, *Eukaryotic Antimicrobial Peptides: Promises and Premises in Food Safety.* Journal of Food Science, 2006. **71**(9): p. R125-R135.

74. Alexander, T.E., L.D. Lozeau, and T.A. Camesano, *QCM-D Characterization of Time-Dependence of Bacterial Adhesion.* The Cell Surface, 2019: p. 100024.

75. Hicks, R.P., et al., *De novo design of selective antibiotic peptides by incorporation of unnatural amino acids.* Journal of medicinal chemistry, 2007. **50**(13): p. 3026-3036.

76. Deslouches, B., et al., *De novo generation of cationic antimicrobial peptides: influence of length and tryptophan substitution on antimicrobial activity.* Antimicrobial Agents and Chemotherapy, 2005. **49**(1): p. 316-322.

77. Brogden, N.K. and K.A. Brogden, *Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals?* International journal of antimicrobial agents, 2011. **38**(3): p. 217-225.

78. Chen, Y., et al., *Rational design of α-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index.* Journal of biological chemistry, 2005. **280**(13): p. 12316-12329.

79. Jiang, Z., et al., *Effects of net charge and the number of positively charged residues on the biological activity of amphipathic α-helical cationic antimicrobial peptides.* Peptide Science, 2008. **90**(3): p. 369-383.

80. Lozeau, L.D.R., Marsha W; Camesano, Terri A, *A QCM-D study of the concentration- and time-dependent interactions of human LL37 with model mammalian lipid bilayers.* 2018: p. Under review.

81. Wang, K.F., R. Nagarajan, and T.A. Camesano, *Antimicrobial peptide alamethicin insertion into lipid bilayer: a QCM-D exploration.* Colloids and Surfaces B: Biointerfaces, 2014. **116**: p. 472-481.

82. Alexander, T.E., Lozeau, Linsday D., Camesano, Terri A., Hoy, Frank, *Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program.* Portland International Center for Management of Engineering and Technology (PICMET), 2019.

83. Kučerka, N., M.-P. Nieh, and J. Katsaras, *Fluid phase lipid areas and bilayer thicknesses of commonly used phosphatidylcholines as a function of temperature.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2011. **1808**(11): p. 2761-2771.

84. Voinova, M.V., et al., *Viscoelastic acoustic response of layered polymer films at fluid-solid interfaces: continuum mechanics approach.* Physica Scripta, 1999. **59**(5): p. 391.

85. Arouri, A., M. Dathe, and A. Blume, *Peptide induced demixing in PG/PE lipid mixtures: A mechanism for the specificity of antimicrobial peptides towards bacterial membranes?* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2009. **1788**(3): p. 650-659.

86. Mihajlovic, M. and T. Lazaridis, *Antimicrobial peptides bind more strongly to membrane pores.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2010. **1798**(8): p. 1494-1502.

87. Shahmiri, M., et al., *Membrane core-specific antimicrobial action of cathelicidin LL-37 peptide switches between pore and nanofibre formation.* Scientific reports, 2016. **6**: p. 38184.

88. Uzarski, J.R., et al., *The effects of solution structure on the surface conformation and orientation of a cysteine-terminated antimicrobial peptide cecropin P1.* Colloids and Surfaces B: Biointerfaces, 2008. **67**(2): p. 157-165.

89. Peterson, A.A. and E.J. McGroarty, *High-molecular-weight components in lipopolysaccharides of Salmonella typhimurium, Salmonella minnesota, and Escherichia coli.* Journal of Bacteriology, 1985. **162**(2): p. 738.

90. Mason, A.J., et al., *Membrane interaction of chrysophsin-1, a histidine-rich antimicrobial peptide from red sea bream.* Biochemistry, 2007. **46**(51): p. 15175-15187.

91. Tripathi, A.K., et al., *Identification of GXXXXG motif in Chrysophsin-1 and its implication in the design of analogs with cell-selective antimicrobial and anti-endotoxin activities.* Scientific reports, 2017. **7**(1): p. 3384.

92. Esposito, S., S. Noviello, and S. Leone, *Epidemiology and microbiology of skin and soft tissue infections.* Current opinion in infectious diseases, 2016. **29**(2): p. 109-115.

93. Emoto, K., J.M. Harris, and J.M. Van Alstine, *Grafting poly (ethylene glycol) epoxide to amino-derivatized quartz: effect of temperature and pH on grafting density.* Analytical chemistry, 1996. **68**(21): p. 3751-3757.

94. Pasternack, R.M., S. Rivillon Amy, and Y.J. Chabal, *Attachment of 3-(aminopropyl) triethoxysilane on silicon oxide surfaces: dependence on solution temperature.* Langmuir, 2008. **24**(22): p. 12963-12971.

Supplementary Data

PEG deposition through QCM-D

The masses of PEG deposited in nanograms at temperatures ranging from 23^{o}C to 55^{o}C and high and low salt concentrations are outlined below in Figure 1 for PEG 7500. There was statistically more PEG 7500 deposited for the low salt condition for every temperature tested as compared to at 23C as reported in the previous study [2]. For the high salt concentration there was not statistically significant difference between any of the temperature conditions and the high salt control at 23C. For the low salt concentration there was a trend of increasing PEG deposition as temperature increase as expected based on the literature[93], however there was no statistical significant difference between the amount of PEG 7500 deposited between these higher temperature conditions. This led to the decision to pick 37^{o}C and 55^{o}C of the low salt condition as the

temperatures at which to test antimicrobial activity. A minimum of 5 replicates was gathered for each low salt condition and high salt condition. Standard error was used for error bars.

PEG 7500 density at 23 ºC low salt was 2.46±2.64 ng (based on Lozeau *et al.* [2]), at 37 ºC was 23.8±8.97 ng, at 45ºC was 37.8 ±25.0 ng, at 50ºC was 55.4±37.2 ng, and at 55ºC was 67.6±25.1 ng. At high salt, PEG 7500 density at 23 ºC was 2.07±0.97 at 37 ºC was 2.40±1.54 ng, at 45ºC was 3.65±1.57 ng, at 50ºC was 0.21±0.12 ng, and at 55ºC was 23.2±8.10 ng.

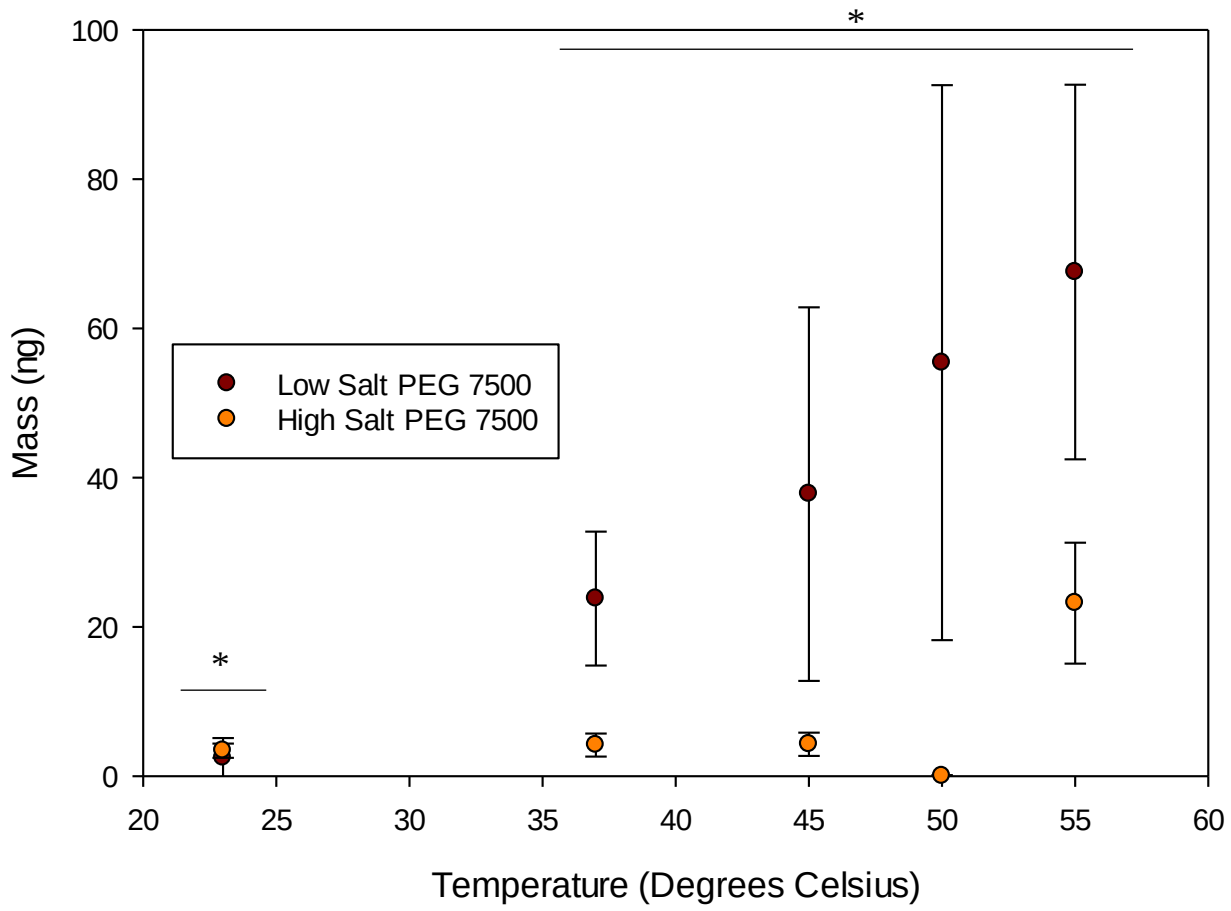


Figure 1: PEG 7500 deposition in nanograms. Salt concentration and PEG incubation temperature were varied. For each condition n.>5 replicates was performed. Data was obtained using the QCM-D. For the low salt concentration there was a statistically significant increase in PEG 7500 deposition between PEG 7500 at 23C versus the higher temperature conditions. There was no significance between the higher temperatures.

For the high salt condition there was no statistical difference between conditions. One way anova using a Tukey test was performed statistical significance $p<0.05$.

Additionally, the amount of PEG in nanograms deposited at temperatures from 23ºC to 55ºC and high and low salt concentrations can be seen below in Figure 2 for PEG 866. A minimum of 5 replicates was gathered for each low and high salt condition. Standard error was used for error bars. There was statistically less PEG 866 deposited for the low salt condition for every temperature tested as compared to at 23ºC as reported in the previous study [2]. For the high salt concentration there was no clear trend as far as increasing temperature. Initially increase temperature increased the deposition at 37ºC and 45ºC but a decreases at 50ºC and 55ºC. This trend is not easily explained and is contrary to what was expected [93]. Compared to 23ºC at the low salt concentration there was a decrease in PEG density at all temperatures for the high salt concentration condition. There was no statistically significant difference between any of the temperature conditions and the high salt control at 23ºC. For the low salt concentration there was a trend of decreasing PEG deposition as temperature increase which is in disagreement with the literature[93], but this may be due to the relatively small size of the molecule in the first place the potential hydrolysis of the APTMS layer [94]. This led to the decision to pick 37C and 55C of the low salt condition as the temperatures at which to test antimicrobial activity in order to compare them to the results for PEG 7500 deposited at 23C since these were the only conditions with a higher PEG density.

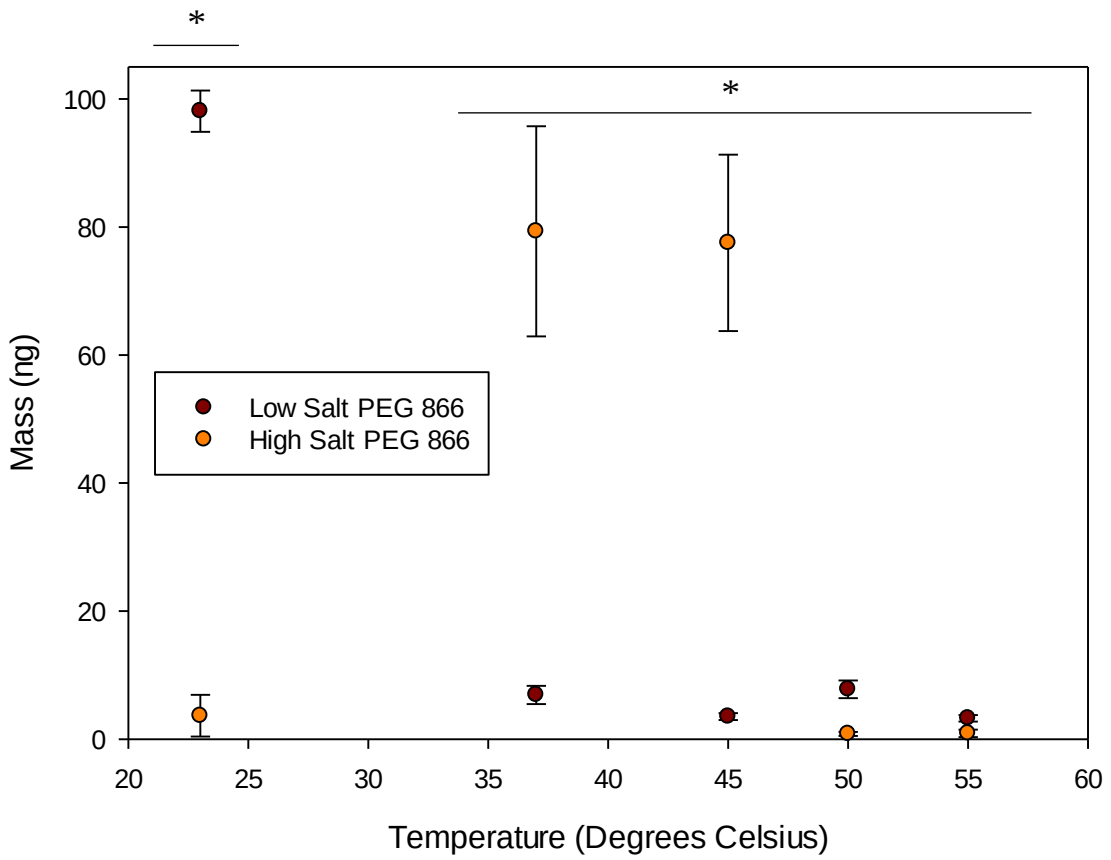


Figure 2: PEG 866 deposition in nanograms. Salt concentration and PEG incubation temperature were varied. For each condition n.>5 replicates was performed. Data was obtained using the QCM-D. For the low salt concentration there was a statistically significant decrease in PEG 866 deposition between 23C and the elevated incubation temperatures. For the high salt condition there was no statistical difference in PEG 866 deposition. One way anova using a Tukey test was performed statistical significance $p<0.05$.

PEG density at 23 ^{o}C low salt was 98.1±7.88 ng (based on previous data), at 37 ^{o}C was 6.90 ± 2.54 ng, at 45^{o}C was 3.52±1.31 ng, at 50^{o}C was 7.78±3.91 ng, and at 55^{o}C was 3.25±1.15 ng. At high salt, PEG density at 23 ^{o}C was 3.65±3.25 at 37 ^{o}C was 64.2±16.4 ng, at 45^{o}C was 61.1±41.3 ng, at 50^{o}C was 0.47±0.31 ng, and at 55^{o}C was 0.90±0.59 ng.

The thickness of the C-CHY1 brush layer was calculated using the Voight-Kelvin viscoelastic model. As expected the thickness of each layer increased at the PEG 7500 incubation increased. This is in agreement with the trend for PEG 7500 mass deposition. More PEG on the surface leads to thicker brush layers (cite). Just as with the PEG mass deposition there was no statistical difference between the higher temperatures PEG 7500 brush thicknesses. There was a statistically higher C-CHY1 brush layer thickness between the PEG 7500 deposition at 55C versus 23C. This suggest a denser more hydrated PEG layer and thus an increase in the number of binding sites for C-CHY1. The thickness of the layers is 21.9nm, 29.2nm, and 40.3nm for the C-CHY1 brush layers formed with PEG deposited at 23C, 37C, and 55C respectively.

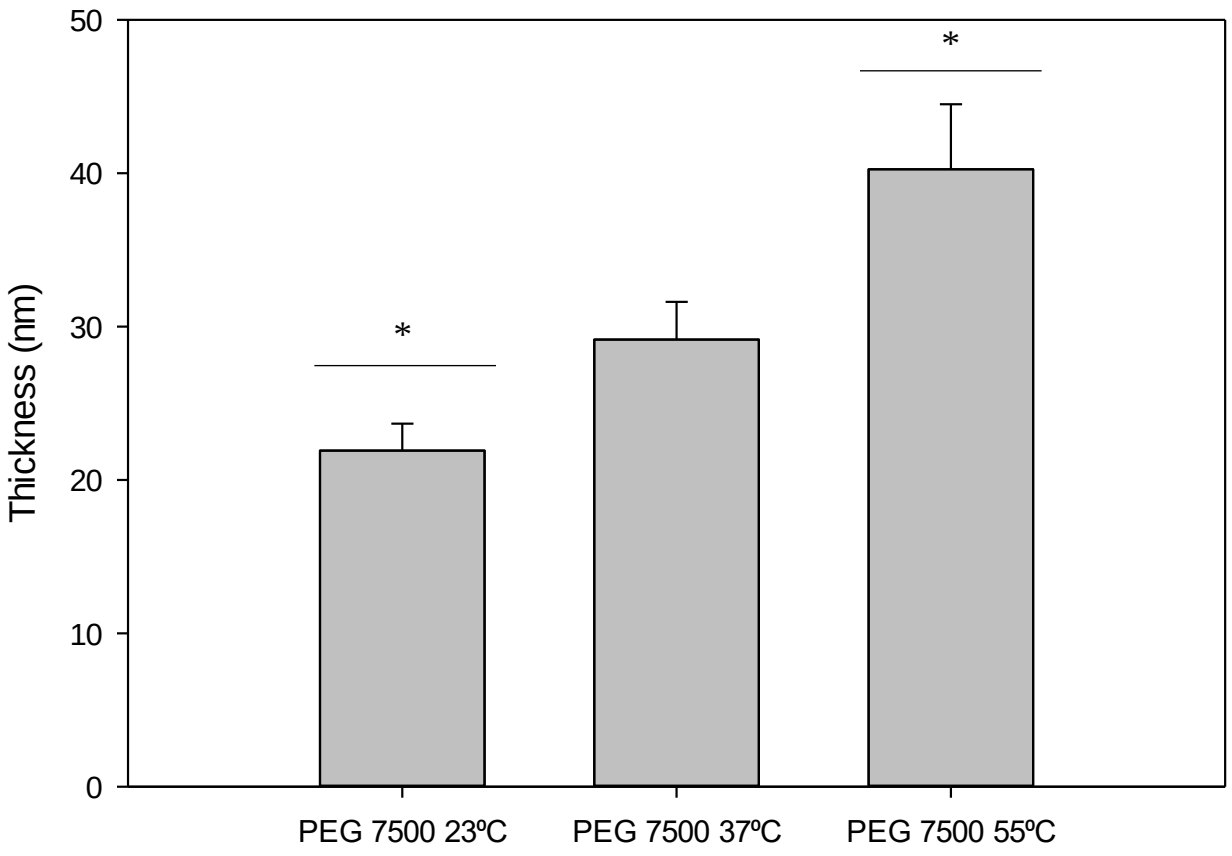


Figure 3: C-CHY1 layer thickness versus PEG 7500 incubation temperature n>7. There was a statistical difference between the thicknesses of the C-CHY1 brush layer when the PEG 7500 is incubated at 23C versus

55C. There was no statistical difference between the thickness of the C-CHY1 brush layer when the PEG 7500 is incubated at 23C versus 37C or between 37C and 55C. One way anova using a Tukey test was performed statistical significance $p<0.05$.

Antimicrobial Activity

The antimicrobial activity was determined for each brush system for *E.coli* and *S.aureus* at bacterial incubation temperatures of 23ºC and 37ºC. For *E.coli* the killing percent was not significantly improved compared to the PEG incubation and bacterial incubation at 23ºC. There was a statistically significant difference between the E.coli killing when the PEG 7500 was introduced and incubated at 23ºC versus 37ºC and when the PEG 7500 was introduced and incubated at 55ºC comparted to 55ºC. There was a statistically significant difference between the E.coli when incubated at 23ºC versus 37ºC in the condition where PEG 7500 was introduced and incubated at 55ºC. See Figure 4. For the S. aureus there was no statistical difference between the different PEG 7500 incubation temperature groups. There was however a statistical difference within each group for S. aureus based on bacterial incubation temperature with a statistically significant increase in S. aureus killing when incubated at 37ºC. See Figure 4.

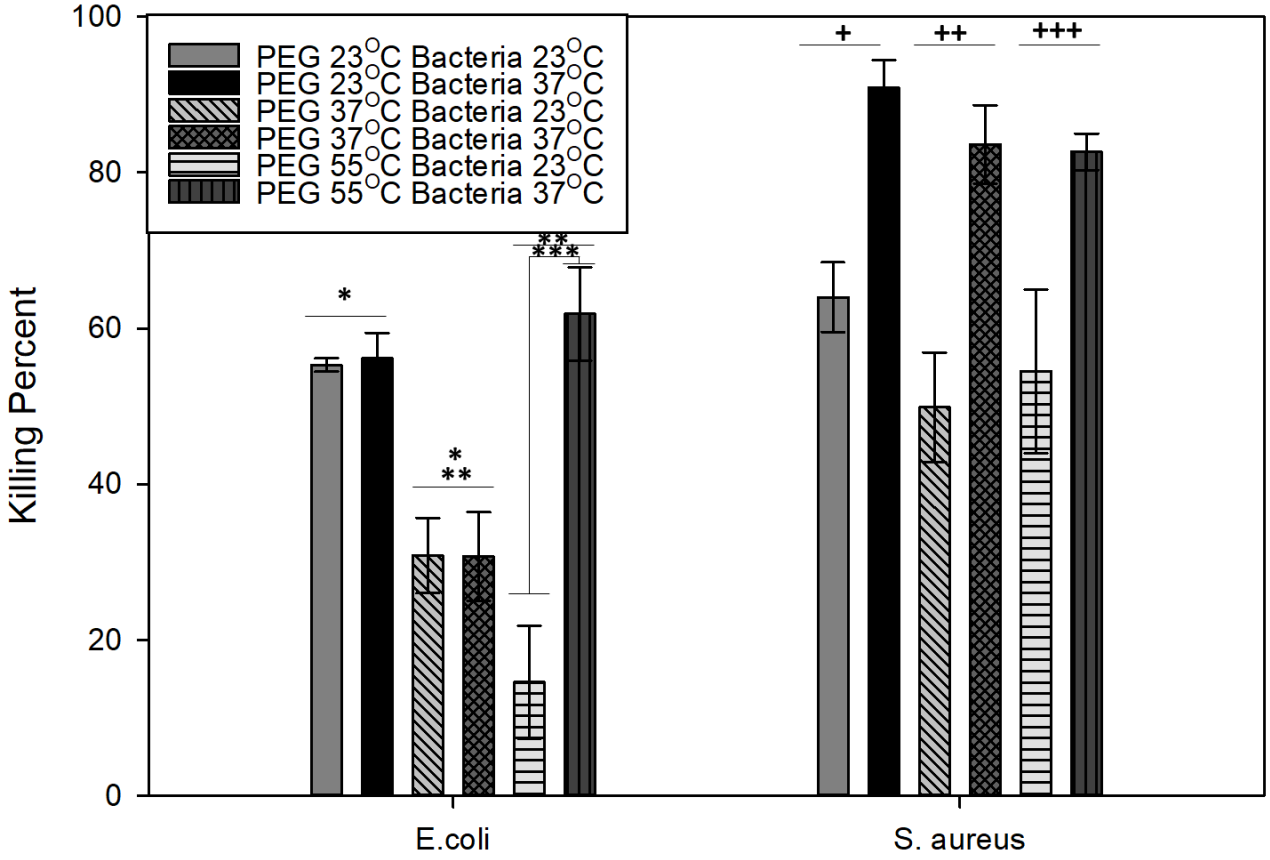


Figure 4: Bacterial Killing vs PEG 7500 density and Bacterial Incubation Temperature. For each condition n.>5 replicates was performed. Data was obtained using the QCM-D and live-dead staining. For *E.coli* there was a statistically significant decrease in bacterial killing from PEG 7500 incubation at 23^{O}C and bacterial incubation at 23^{O}C compared to PEG incubation at 37^{O}C and bacterial incubation at 37^{O}C. For *E.coli* as PEG incubation temperature increases bacterial efficacy decreases. For *S. aureus* there is a statistically significant decrease between in antimicrobial activity between PEG 7500 incubation at 23^{O}C, 37^{O}C, and 55^{O}C for bacterial incubation temperature at 23^{O}C versus 37^{O}C. Two way anova using a Tukey test was performed statistical significance $p<0.05$.

Representative Frequency and Dissipation Trends for Gram-positive and Gram-negative Supported Lipid Bilayers

Supported lipid bilayers were formed as described in the Methodology section. Data was collected using the Q-Sense E4 (Biolin Scientific, Sweden). Frequency and dissipations data is collected in real time. Below are figures representative of the changes in frequency and dissipation throughout the whole experiments from vesicle deposition, vesicle rupture, SLB formation, and peptide interactions. Data is shown for the 3rd through 11th overtones.

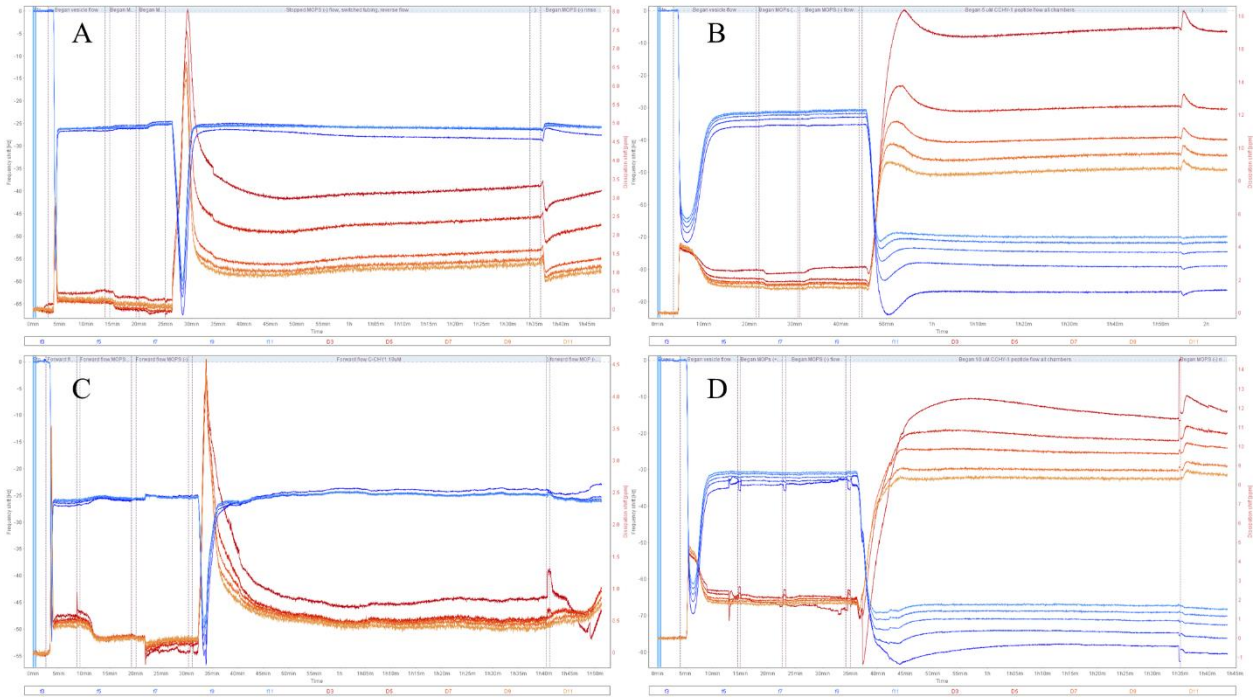


Figure 5: Representative frequency and dissipation changes for Gram-positive SLBs. Frequency and dissipation data for the 3rd through 11th overtones are displayed, data was obtained using the QCM-D. Frequency is in blue and dissipation in red. A) For PCPG at 23^{o}C. B) For PCPGLTA at 23^{o}C. C) For PCPG at 37 ^{o}C. D) For PCPGLTA at 37 ^{o}C. For all conditions n>4 replicates.

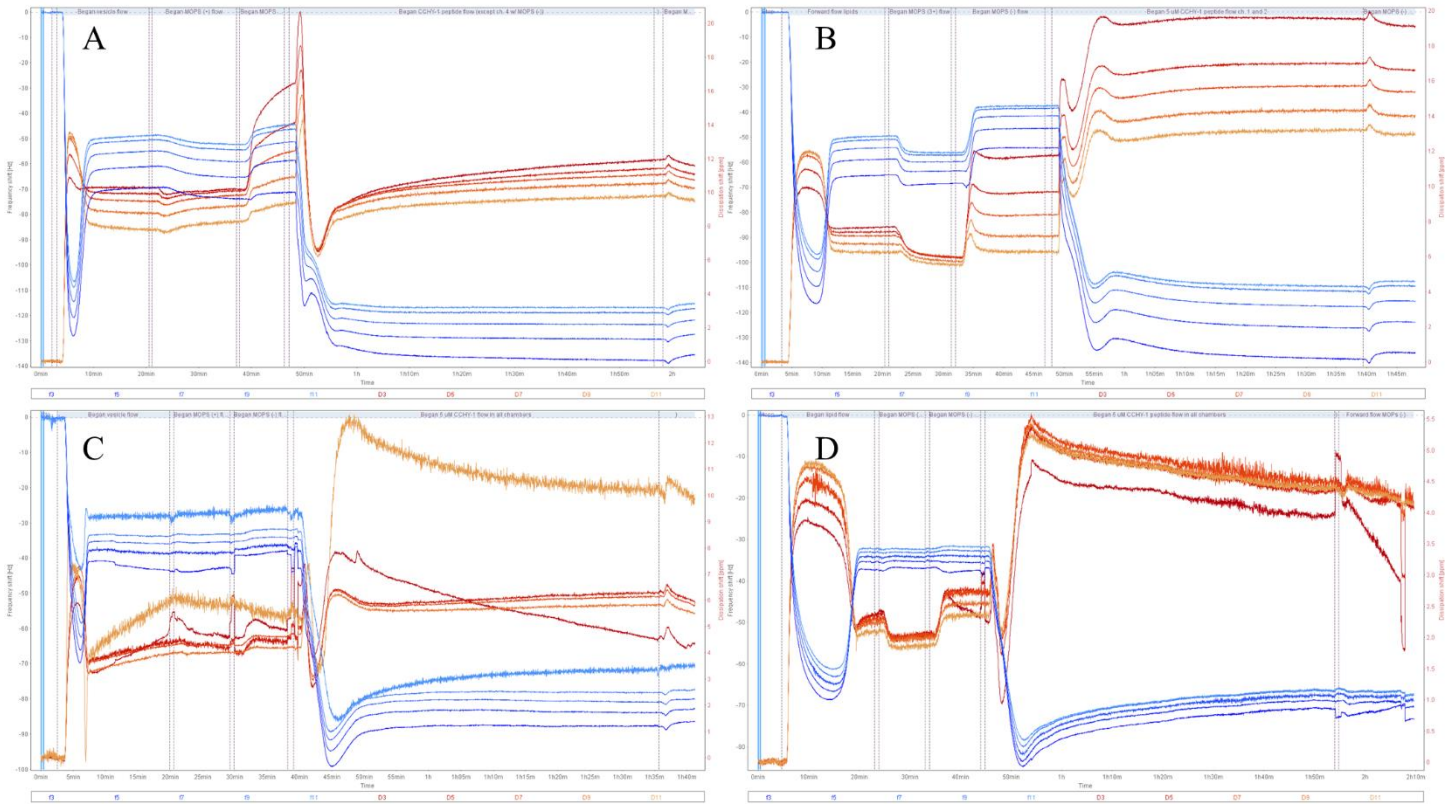


Figure 6: Representative frequency and dissipation changes for Gram-negative SLBs. Frequency and dissipation data for the 3rd through 11th overtones are displayed, data was obtained using the QCM-D. Frequency is in blue and dissipation in red. A) For PEPG at 23ºC. B) For PEPGLPS at 23ºC. C) For PEPG at 37 ºC. D) For PEPGLPS at 37 ºC. For all conditions n>4 replicates.

Specific Aim 3: Chapter 6 Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program

Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program

An entrepreneurial mindset can be defined as "the inclination to discover, evaluate and exploit opportunities" [1], or the mindset of a "habitual entrepreneur" [2]. There are many other definitions of the term however the key concept captured is the ability to take advantage of opportunities, commercialization [1, 2]. When developed during practically oriented entrepreneurship courses mindset encourages the increase in entrepreneurial intentions, desire to form a start-ups, which is essential for economic growth and the translation of new ideas [3-5]. This education is important as students are more than twice as likely to start a company compared to tenured faculty [5, 6]. Additionally the learning of entrepreneurial concepts and skills contributes to entrepreneurial success [7-11]. This demonstrates the importance of instilling an entrepreneurial mindset which provides the ability to recognize opportunities for entrepreneurship making commercialization efforts more likely and more successful [1, 2, 7, 10, 11]. Exposure to entrepreneurship via education leads entrepreneurial mindset and is an important marker for entrepreneurial success, understanding the best practices for teaching entrepreneurship is vital [1, 2, 5, 7]. Data collected through WPI's NSF-funded *IGERT: Training Innovative Leaders in Biofabrication*, PhD training program, was analyzed in order to study the best ways to instill an entrepreneurial mindset in graduate students [12].

***Abstract*— In 2013, Morris, Kuratko and Cornwall [13] reported a trend in entrepreneurship education moving from a focus on starting small businesses toward stimulating innovative, growth-oriented ventures. They forecast a broadening of entrepreneurship education leading students to "think and act in entrepreneurial ways in all facets of life, to pursue careers where entrepreneurship can manifest in many different ways, and to apply the entrepreneurial mindset in their personal lives" (p. 16). This approach was adopted at Worcester Polytechnic Institute (WPI) in the design of a Ph.D. program for life science and bioengineering students. The faculty instigators recognized that the majority of STEM doctoral program graduates were not choosing academic careers and were not necessarily being prepared to engage in entrepreneurial behavior either through new venture creation**

or opportunity seeking within larger organizations. In reviewing the entrepreneurship education literature, it was noted that many programs lacked clear learning objectives and that few provided outcome measures [14, 15]. In this paper, we explain how learning outcomes were developed and implemented for a program selected by the National Science Foundation for its innovativeness and potential for replication, and how results have been and are being evaluated in accordance with Mialaret's [16] theoretical perspective for designing and implementing an education program.

6.1 Introduction

Anticipating the skills that our graduates will need to serve them throughout their careers is a challenge. As stated by former US Secretary of Education Richard Riley, we are preparing students for jobs that do not yet exist [17]. Having a firm grounding in the field of entrepreneurship with an emphasis on self-employment, innovation, and invention may be the best way for science students to prepare for the 21st century workforce, particularly as employment prospects in STEM disciplines become increasingly uncertain [18]. In fact, students who are exposed to entrepreneurship during their training are three times more likely to start their own business and/or be self-employed, have 27% higher incomes, and report being more satisfied with their jobs [19]. Beyond the most apparent goals of self-employment and starting a business, researchers have identified a number of other advantages of teaching students about entrepreneurship, even if business creation is not the primary goal. For example, Blais [20] stated that teaching entrepreneurship as a curriculum component for all students will add a practical basis for both the students and faculty, foster creativity and innovation, improve teamwork, introduce the concept of small business ownership, foster an appreciation of how start-ups develop the regional and local economies, and become better at intrapreneurship. This last point can be relevant for graduates pursuing any type of job, including in large corporations [21] regardless of their interest in self-employment.

Although the marriage between entrepreneurship and STEM in academic disciplines may not seem obvious or easy to implement, there are numerous reasons why these disciplines are naturally suited for pairing. For example, scientists and engineers who are trained in entrepreneurship will become better at

understanding the societal impacts of their work, which can make them more motivated to pursue scientific and technical advances, and will translate well to their technical disciplines [21].

Wheeler [22] found that more science graduates formed their own businesses than business graduates, at rates of 47% and 35%, respectively. Graduates of programs with entrepreneurship in their titles started ventures more than graduates with any other degrees [23]. When graduates of STEM disciplines start businesses, those ventures tend to be related to their technical discipline. For example, most engineering graduates who start businesses choose to develop ventures related to consulting, manufacturing, or information technology [24]. According to the AUTM US Annual Licensing Activity Survey [25], universities in the United States generated a record number of start-up companies with the help of their technology transfer offices in 2017 representing a 32% increase over the previous five years.

While there is evidence of entrepreneurial behavior emerging from STEM disciplines, scholars and practitioners have called for more entrepreneurship education on the assumption that such education could lead to higher levels of venture creation and commercial licensing, in turn positively impacting economic development [26-28]. Nevertheless, barriers exist at the faculty, student and institutional levels to teaching entrepreneurship in STEM disciplines. In this paper, we provide a case study of a doctoral degree initiative supported by the National Science Foundation (NSF) through their Integrated Graduate Research Traineeship program designed to prepare doctoral students receiving STEM-related educations to engage in entrepreneurial behavior as venture creators and organizational innovators both as students and graduates. From 1998 through 2013, the NSF conducted competitions for financial support through IGERT. Grants were awarded to 278 academic programs proposing to create innovative, evidence-based traineeship approach in high-priority interdisciplinary research areas. The goal of IGERT was to establish new models for graduate education and training. Such models were expected to cross disciplinary boundaries, engaging students in the processes of translating innovations for societal benefit. Outcome assessment was required. At WPI, analysis of detailed documentation of the program implementation and outcome measurements led to the identification

of strategies and tactics capable of overcoming barriers and achieving desired results. Although the program example is at the Ph.D. level, we believe it has implications for STEM-focused entrepreneurship at the levels of undergraduate and masters degrees. A specific objective of this program was to spread entrepreneurship education across the curriculum, preparing students for their future careers.

6.2 Growth and Current state of entrepreneurship Education

Researchers have noticed a vast increase in entrepreneurship programs in universities during the latter part of the 20th century. For example, the number of undergraduate entrepreneurship courses in Canadian universities increased by 450% in a two-decade period [29, 30]. In the U.S., Vesper and Gartner [31] recognized an increase from 85 programs offering entrepreneurship in the 1970s to 383 by the end of the 1990s, with over 500 majors, minors, and certifications being in place early in the 21st century [32]. By 2016, entrepreneurship was reported being taught in over 3,000 universities [14]. Similar trends were observed in Europe [29].

Entrepreneurship education encompasses multiple aspects such as courses in business and in other disciplines that include entrepreneurship content, consulting opportunities, student project work, and case study writing [33]. It also involves experiences outside of the classroom in start-ups (either as interns or as students starting new ventures) and participation in preexisting business or startup-specific training programs, such as through NSF I-Corps (Innovation Corps, National Innovation Network Teams Program) [34-36]. Other opportunities include private or university-based business incubators, with such well-known programs as 1871- Chicago's Technology & Entrepreneurship Center and the Uppsala Innovation Center in Sweden, as well as networking events and guest speakers. Specific topics may include opportunity recognition, formation of a team, commercialization, translation, resource allocation, risk management, as well as business development, finance, and marketing [37]. Collectively, these activities are designed to provide students with the ability to recognize commercial opportunities and gain the knowledge and skills needed to move ventures forward, but require a distinct educational model that has been difficult for institutions to create.

It is only in recent years that the academic community has recognized that certain cognitive skills could distinguish between successful entrepreneurs and either novices or non-entrepreneurs [38]. Duening [38] focused on the five "minds" approach to demonstrate which cognitive skills are necessary to include in a curriculum on entrepreneurship. His approach focuses on 1) The Opportunity Recognizing Mind, 2) The Designing Mind, 3) The Risk Managing Mind, 4) The Resilient Mind, and 5) The Effectuating Mind. Duening [38] stated that focusing on these five minds can provide the intellectual foundation around which curricula can be developed, and reviews literature for each of these areas. This desire to put entrepreneurship curricula into an intellectual framework is counter to early attempts to identify a personality type that leads to successful entrepreneurship.

According to Duening [38], attempts to identify personality traits, characteristics, or even behaviors that lead to success or that define entrepreneurs could not be identified. Fortunately, there was success when researchers began focusing on how entrepreneurs think. Mitchell *et al.* [39] were able to identify differences in how successful entrepreneurs make judgments and decisions, compared to non-entrepreneurs. This research laid a foundation upon which Duening [38] could establish his approach of focusing on 5 cognitive principles, or minds. In contrast, Turner *et al.* [40] argued that while entrepreneurs are known for their creativity and ability to deal with uncertainty and risk, STEM students are generally risk-adverse. Thus, they suggested that entrepreneurship programs should instead focus on attracting students with some innate entrepreneurial attributes. Fiet [41] explained that in order to master entrepreneurial skills and knowledge, students must have these topics fully integrated into their curricula. While there is some disagreement, there is a large amount of support for the notion that entrepreneurship can be taught and learned [42, 43].

6.3 Barriers to Implementation of Entrepreneurship in STEM Education

While the teaching of entrepreneurship is one of the fastest growing areas of the undergraduate curriculum [44], with an estimated >5,000 entrepreneurship courses being offered in the U.S. as of 2008 [32], large numbers of STEM curriculums at colleges and universities do not regularly include an entrepreneurship component.

6.3.1 Faculty and Institutional Barriers

For STEM faculty that want to teach entrepreneurship, barriers to inclusion of these concepts in their courses and projects revolve around expertise, resources, and incentives. Tightly structured degree programs may offer little flexibility for students to choose entrepreneurship classes as electives [3], [44]. There has also been a problem in finding qualified faculty to teach entrepreneurship classes. In 1999, Brown noted that North American Universities had trouble filling entrepreneurship chair positions [45]. By 2004, Brush *et al.* [46] echoed that the demand for courses in entrepreneurship far exceeded the supply of doctoral-trained professors. A 2012 study identified that it is difficult to find qualified faculty because typical doctoral programs emphasize narrow specialization in a research field. Entrepreneurs are interdisciplinary-skilled individuals who may choose to prioritize consulting over publishing, and practice over research [47]. For STEM faculty who are interested in teaching entrepreneurship, there could be concerns about how this topic fits within their tenure and promotion goals. Institutions expect STEM faculty to be experts in their discipline, and such expertise is demonstrated by presenting at national conferences and publishing in highly ranked journals in their fields. It is unclear whether incorporating entrepreneurship into STEM courses or creating new courses supports the professor's long-term development or the institution's prestige.

STEM professors need information on how to take resources that are available in other fields, such as literature from the business school or industrial engineering case studies, and be able to adapt or integrate those resources to their own courses. While there are textbooks, case studies, and course modules available on entrepreneurship, STEM faculty may struggle with adapting this to their own disciplinary courses. In addition, faculty may need guidance on how to teach entrepreneurship, as the techniques for teaching the scientific method may differ from the methods for teaching a business case. Collectively, the limited resources and incentives, combined with limited personal experience, create significant barriers for STEM faculty to teach entrepreneurship concepts, either in coursework or through projects [13]. For faculty, it can be a challenge to determine the best type of model for use in entrepreneurship courses. Fiet [41], [48] expressed a

concern that many of the models and cases being employed are not based in theory, and they are overly based on practical experience or empiricism, rather than well-constructed intellectual models. Numerous efforts are taking place to help faculty learn about how to teach entrepreneurship within a STEM context, such as those by the Kern Family Foundation, the Coleman Foundation Faculty Entrepreneurship Fellows program, and the Experiential Classroom run out of the Entrepreneurship and Innovation Center at the University of Florida [49].

At the institutional level, there are sometimes administrative barriers to expanding the reach of entrepreneurship into STEM disciplines, especially the concern of determining where entrepreneurship fits within the academic curriculum. There is probably no single solution that would satisfy all types of universities, but isolating entrepreneurship in either the business school or a STEM discipline does not seem to be as fruitful as some form of interdisciplinary cooperation and collaboration between units. Gibb [50] has pointed out that there are multiple types of activities being pursued under the banner of entrepreneurship education, yet few studies have undertaken extensive evaluations of entrepreneurship programs [51]. Further study and evaluation of entrepreneurship programs in academic institutions are needed. In turn, we may see a shift in more institutions from empirical approaches to those based in pedagogical models.

Some of these challenges are exacerbated in graduate curricula, since many graduate programs operate with a high degree of autonomy where it is assumed that the individual departments should address the graduate student curricular and developmental needs. Further, success of graduate students towards degree completion is attributed mainly to academic background and research skills [52]. There are some indications that the attitude towards exposing graduate students to entrepreneurship is changing. Evidence is being compiled, such as the study by Fayolle *et al.* [53], showing that students who did not previously envision an entrepreneurial career had an increased level of intention to start an entrepreneurial career after exposure to an entrepreneurship education program. Maresch *et al.* [54] also found that entrepreneurial intention was increased in engineering students exposed to entrepreneurial education. In particular programs such as The

Lean Startup [55] may be more successful due to the foundation of the empirical circle with which engineers are familiar [54].

6.3.2 Student Barriers

One issue affecting student participation in learning about entrepreneurship is difficulty scheduling courses, given limited flexibility within their major concentrations [15]. Often, STEM programs require a large number of credit hours for graduation, and the programs may be highly structured already [56]. Additionally, students may not fully understand what entrepreneurship is and, therefore, may not grasp how entrepreneurship may fit into their own professional career development and opportunities.

Alternatively, there is some evidence of student demand for entrepreneurship in STEM education. A survey of 501 senior-level engineering undergraduates at three different universities in the US found that 69% of students agreed that entrepreneurship could broaden their career opportunities and provide them with more professional choices [57]. This statistic rose to 82% for a subsample of students who had taken at least one course in entrepreneurship.

In the following sections, we offer the WPI IGERT program as an example of a proactive effort to reduce barriers and identify strategies for infusing entrepreneurship education into STEM disciplines. Although the program was implemented at the doctoral level, the practices and outcomes have stimulated cooperation between business and STEM educators in WPI's masters and undergraduate level education.

6.4 Methods

Barriers, initiatives, activities and outcomes discussed in this paper were compiled from participant observation by the authors, and formative and summative evaluations undertaken as part of a National Science Foundation (NSF)-funded training grant *IGERT: Training Innovative Leaders in Biofabrication* at Worcester Polytechnic Institute (WPI) [58]. The authors consist of the principal investigator and a co-principal investigator of the IGERT grant and two of the inaugural students in the doctoral program. The IGERT grant required meticulous compilation and reporting of data documenting the development and implementation of

the program, its progress, and results achieved. In the early years of the program, an external evaluator was employed to measure program impacts. Subsequently, the principal and co-principal investigators were charged with assessment and reporting.

Metrics were comprehensive, providing data on program inputs, activities and outcomes. Examples included student recruiting practices and results, research grant proposal submission and approval, publications and presentations, participation in competitions, internships obtained, placement upon graduation, and more. Data were analyzed by the co-authors of this paper, additional co-principal investigators, and the external reviewer. The principal investigator and co-principal investigators all approved each report submitted to the National Science Foundation over the period covered by the IGERT grant. Benchmarks and recommendations extracted from the reports and described in this paper are the responsibility of the authors.

The framework for the analysis of the WPI case was derived by Baptista and Naia [59] and based on Mialaret's [16] questions for designing and implementing an education program. Mialaret proposed that five questions should be answered to provide the foundation for a successful program: Why, What, For Whom, How, and For Which Results. Fayolle and Gailly [53, 60] suggested reordering the questions in order to better define the audience and the teaching model. We adhere to the Fayolle and Gailly approach in our analysis of the WPI IGERT program:

- *Why*, i.e. what are the objectives?
- *For Whom,* i.e. who is the audience?
- *For Which Results*, i.e. how should the program be evaluated?
- *What*, i.e. what content should the program contain?
- *How*, i.e, what methodologies should be followed?

6.5 Results

“IGERT has been the National Science Foundation's flagship interdisciplinary training program, educating U.S. Ph.D. scientists and engineers by building on the foundations of their disciplinary knowledge with interdisciplinary training” [61]. Subsequent evaluations of IGERT programs reported successful implementations of interdisciplinary education and positive results of student learning outcomes, employment upon graduation, and recommendations of program continuation [62, 63].

6.5.1 Why Develop an Interdisciplinary Education Program for STEM Students?

“The goal of the IGERT program at WPI is to produce a diverse group of future leaders who are able to translate basic discovery research into innovative solutions to unmet medical needs in our global society” [52].

The goal of the IGERT program at WPI is to produce a diverse group of future leaders who are able to translate basic discovery research into innovative solutions to unmet medical needs in our global society” [64].

Objectives for graduates of the program are for the emerging leaders to possess an entrepreneurial mindset and to engage in the practice of their science and engineering education understanding how to translate a discovery or technological advance in the laboratory to acceptance in the marketplace. There are learning objectives for each student related to the student’s specific STEM major and extending into business disciplines regarding the skill to identify commercial opportunities for their research projects and the knowledge to exploit the opportunities through venture creation, licensing and other strategies. There is also the expectation that there will be positive socio-economic [62] results from translating the research from the laboratories to practical application.

The principal investigator and co-principal investigators formed a strategic management team to define objectives and monitor the program from a strategic management perspective. In addition to the learning objectives regarding technical knowledge and translation skills, the leadership group sought opportunities for

trainees to transition through the program in cohorts. In this way, they could learn the value one another's disciplinary specialization and potentially identify cooperative initiatives.

6.5.2 For Whom Will the Program Be Designed and Offered?

Looking specifically at entrepreneurship education, Fayolle and Gailly [53, 60] suggest there are three audiences: entrepreneurs or professionals who seek to acquire skills, entrepreneurial individuals who can benefit from being prepared with entrepreneurial mindsets, and professors and researchers who need a grasp of theory. The primary audience for WPI's Biofabrication Ph.D. program was students who could benefit from an entrepreneurial mindset as they developed intellectual property with potential for commercialization.

The students also had access to programs targeting entrepreneurs and professionals. At approximately the same time that the IGERT program was launched, WPI introduced the Tech Advisors Network, a mentoring program designed for teams desiring to spinoff technology-based ventures from the university. These teams typically consisted of students, faculty, and/or alumni. The Office of Intellectual Property and Innovation at WPI, which has responsibility for technology transfer at the university, also received a grant from the National Science Foundation to serve as a venue for Innovation Corps (I-Corps) programs, thus further offering skills training for teams with venture creation potential.

Additionally, the university recognized that professors in disciplines other than business needed to be introduced to entrepreneurship theories. Faculty sought and were awarded grants from the Coleman Foundation and from the Kern Family Foundation to institute programs preparing non-business professors to incorporate entrepreneurship concepts in classes targeting their respective undergraduate and graduate majors.

The target audience for the IGERT program at WPI was STEM doctoral students. WPI offers additional programs that complement the education the IGERT trainees were receiving, but those programs are primarily directed toward other audiences.

6.5.3 For Which Results is the Interdisciplinary Education Program Evaluated?

One co-principal investigator was assigned as point person for the evaluation process. Additionally, a contract was executed with an external professional evaluator. The National Science Foundation provided a

template for grant recipients to report activities and results. Components expected to be reported include: major activities, specific objectives, significant results, and key outcomes or other achievements. Information was expected to be submitted about opportunities for training and professional development, how the results are being communicated, and future plans for goal accomplishment. Grant recipients were also expected to report on the impact of their programs on various stakeholders and regarding problems encountered and handled [65].

The external evaluator conducted a survey of students upon their acceptance into to program. The survey provided pre-training information regarding student expectations, specifically what they expected to gain from the program. Students reported their expectations regarding learning about other disciplines, working with colleagues from other disciplines, attitude toward business education, translational aspects of research, and more.

An objective of the program was also to foster an environment of collaboration among the student trainees. Annual boot camps were conducted in which the trainees worked in teams not only to solve problems, but also to bond socially. Subsequent interviews conducted by the program evaluator confirmed that the trainees valued each other as individuals and also learned to bring one another's talents and skills into performing the assigned tasks.

In addition to courses within their major disciplines, there were courses required for all IGERT trainees, two in the School of Business and two in the Department of Biomedical Engineering cross-listed with the School of Business. The courses were designed to provide both technical knowledge and translational skills and enabled cross-collaboration with business school doctoral students. Instructors were expected to state specific learning objectives in their course syllabi. In the Business School entrepreneurship and innovation course, students acquired knowledge of underlying theories of innovation and demonstrated that knowledge at the end of the course based on their ability to define key terms, identify major contributors to theory, followed by identifying examples in practice where theory had been applied. For the Business School's

technology commercialization course, students studied commercialization cycle models. In the subsequent Biomedical Engineering courses, they prepared SBIR grant proposals to seek funding for the commercial translation of the intellectual property they were developing within their STEM disciplines. At the suggestion of the program evaluator, external reviewers were brought in to assess the students' work. The evaluator developed a scoring rubric for use both by instructors and external evaluators, as would be done in practice. Evaluation criteria included justification, logic/rationalization, cohesiveness of the approach, and writing. The student trainees provided feedback and suggestions for course evaluation rubrics. In addition to student evaluations, the program evaluator conducted a separate survey to gauge the learning experiences of the trainees. At the end of their first year in the program, trainees were queried to determine what they learned about innovation, biofabrication, translation, and interdisciplinarity.

The principal investigator and co-principal investigators were assessed on their performance and accomplishments. They prepared and submitted reports to respective department heads, advisory committees, and the provost's office. Annual reports were also submitted to the National Science Foundation.

There has been extensive documentation of the effectiveness of the IGERT program. These include:

1. Interdisciplinary research achievements
   1.1 Journal article publications by student trainees, often with faculty co-authors. Articles have appeared in such journals as *Advanced Science*, *Journal of Biomedical Materials Research*, *Journal of Visualized Experiments*, and more.
   1.2 Additional grants received from organizations including the National Institutes for Health and the National Science Foundation
2. Education achievements
   2.1 Conference presentations, e.g. developing innovative mindsets presented to the Biomechanics, Bioengineering, Biomechanics conference; the Biomedical Engineering Society; the American Society for Bone and Mineral Research, and more.

2.2 Conducting workshops for WPI faculty, such as a train-the-trainer workshop on value creation and innovation, and value propositions

2.3 Awards received from such groups as the Women's Impact Network

3. Trainee achievements

3.1 Business launches, such as AMProtection

3.2 Research commercialization collaborations, examples include the Massachusetts Medical Device Development Center (M2D2)

3.3 Summer internships, with such organizations as the U.S. Army Research Institute of Environmental Medicine, Novartis Institutes for BioMedical Research, TEI Biosciences, and others

4. International research partnerships for student experience:

4.1 Molecular biology at the University of Cambridge in England.

4.2 Electrically conductive biofibers at Technion in Israel.

4.3 Matrix composition using Raman spectroscopy at the University of Tubingen in Germany, and other international partnerships.

Other accomplishments include competing, placing, and winning various competitions on innovations, business plans, and more. Students and faculty have been subjects of stories in multiple media outlets, such as nationalgeographic.com, the *Worcester Telegram and Gazette*, the National Science Foundation's *Science Nation*, and others. There have been numerous patent applications and awards resulting from the research of the student trainees.

6.5.4 What Content and Theories Will Be Addressed?

The WPI IGERT program is designed to introduce students to biofabrication. The growing field of biofabrication uses biological matter as the basic building blocks to assemble advanced biological tissues for treatments and screenings. Each student trainee completes a doctoral degree within a specific STEM field, but is expected to discover relationships between the STEM field selected and biofabrication. The majors

chosen by students have included biology, biological engineering, biomedical engineering, chemical engineering and others. The students receive an education in the content of the major in which they ultimately receive a degree.

Students are expected to learn both theory and practical applications associated with innovation and an innovation mindset, relating their disciplinary studies to biofabrication, processes for translating intellectual property development to marketplace acceptance, and disposition and procedures for interdisciplinary research.

6.5.5 How Will the Program Be Operated in Order to Achieve its Objectives?

Following an initial social gathering in which new students were introduced to each other and, after the first year, to those with a year or more in the program, the students were put through a five-day boot camp. In the mornings, the focus was on addressing technical challenges in laboratories. The afternoons were devoted to business innovation and entrepreneurial aspects that they would be facing as they progressed in their education.

The principal investigator and co-principal investigators maintained strategic control over the design and function of the IGERT program. Each performed teaching responsibilities, although the mandated courses for all the trainees were the primary responsibility of two of the co-principal investigators, one in Biomedical Engineering and one in Business. A program coordinator was employed to manage technical details such as budgeting and scheduling.

6.6 Lessons Learned

While it is inappropriate to generalize from a single case study, we conclude from the thorough assessments conducted throughout the duration of WPI's IGERT program that certain strategies and tactics have merit that may be worth replication and further testing at other institutions. The experiences of the WPI faculty and students may encourage additional research studies seeking solutions to many of the barriers discussed

previously in this paper. Such studies could draw upon a combination of literature findings and best practices by faculty who are currently teaching entrepreneurship in STEM courses. Ultimately, the best strategies for integrating entrepreneurship are likely to fit a given institution's culture rather than being generic. The WPI case suggests potential research questions (in italics below) for STEM educators to pursue.

6.6.1 What faculty strategies are effective for introducing entrepreneurship concepts to STEM students?

Are there identifiable practices that faculty can adopt to assist them in integrating entrepreneurship concepts into STEM classes? WPI experimented with guest speakers, including other faculty, alumni, and practitioners, who were invited to the classroom to talk about their experiences, providing students with practical advice, and bridging the gap between students and experts. Observing the approaches taken by guest speakers resulted in IGERT faculty member becoming more comfortable teaching the material themselves. At many colleges and universities, faculty have access to entrepreneurship centers that may offer help. For example, some centers have developed modules that can be provided to faculty in STEM disciplines to teach entrepreneurship concepts such as risk/reward, controls, business terminology, funding venture capital, financial statements, and rules and regulations [49, 66-68] [52][53][54] Using pre-existing modules saves time and effort. While there is currently no centralized clearing house, various programs such as KEEN make modules available, and individual universities such as Vanderbilt offer modules for free use [49, 66].

The WPI IGERT program integrated entrepreneurship concepts into selected STEM courses. One example involved introducing entrepreneurship into the Engineering Design Process (EDP). The EDP satisfies the ABET accreditation requirement to have certain design components, including a capstone course or experience. At the beginning of the EDP, students are given a client statement. This causes students to think about the customer's need and beyond that to societal and market needs for the particular technology or product. When students learn to understand how all steps of the EDP are used to work towards a solution, it is easier for them to see how to translate the content of their project into a marketable product or service.

The WPI experience benefited from the Experiential Classroom, the Coleman Foundation Faculty Entrepreneurship program, and the Kern Family Foundation Engineering Entrepreneurship Network as tools for incentivizing the teaching of entrepreneurship concepts. Other sources for STEM faculty to gain exposure to entrepreneurship education include professional societies. Many STEM-based professional societies such as the American Chemical Society (ACS), the Ecological Society of America, and the Wildlife Society have education sections. The annual VentureWell Open Conference has several sessions specifically dedicated to building entrepreneurship curricula for non-business students, creating makerspaces, and teaching the entrepreneurial mindset to STEM students [69].

Looking beyond WPI, immersion learning is becoming increasingly popular among STEM faculty [24, 42, 56]. In the biomedical fields, immersion-type learning often involves visits to clinics or hospitals involving discussions with practicing clinicians to determine their highest needs to recognize opportunities. An example at the graduate level is the Master of Biomedical Innovation and Development (MBID) program at Georgia Tech where students with a variety of undergraduate degrees (business and STEM) gain practical and hands-on experience on the development of medical devices from idea to commercialization [70]. This program is aided by real clinical experience and industrial partners, and has led to well-rounded students prepared for a multitude of careers. In other engineering fields, immersion learning is also called "design-centered" learning and entrepreneurship, which, according to Goldsby *et al.* [71], is an iterative hands-on process of ideation, prototyping, market engagement, and business modeling to establish market opportunity and feasibility.

6.6.2 How might student acceptance of entrepreneurship be gained?

At WPI, STEM faculty reached out to the entrepreneurship center faculty and staff in recognition of the intent at the National Science Foundation to place more emphasis on the commercialization of intellectual property. The overture led to greater communication and coordination between the School of Business and representatives of STEM disciplines. STEM faculty began emphasizing practical and technical concepts

associated with entrepreneurship that were beneficial to students, which in turn triggered increased consideration by students toward self-employment as a career option.

An effective element of the IGERT program was the decision to bring in multiple practicing entrepreneurs from STEM-related ventures demonstrating practical applications of the classroom materials. Students were inspired by hearing directly from entrepreneurs who experienced both successful and unsuccessful ventures.

At WPI, assessments credited formal entrepreneurship coursework, weekly professional development modules, and experience outside of the classroom in the form of either an internship or global research experience with forging entrepreneurial mindsets in the trainees.

One Ph.D. student attributed a shift in his research and career path to IGERT teachings (Figure 1). His project was initially focused on understanding the mechanism of action of tethered antimicrobial peptides as well as how to best optimize antimicrobial activity, performed under the pre-text to prevent food-borne illness outbreaks. Without the IGERT program, the project would have been strictly focused on the fundamental research concepts with a tangential connection to the management of foodborne illnesses. After learning the entrepreneurial mindset early in the IGERT program based on Stauffer's 'Thinking Clockwise' [72], the student considered the project's commercial and societal impact, adhering to the entrepreneurial mindset. Consideration of product-market fit in order to prepare for entrepreneurial pitches as part of the IGERT curriculum led to a value proposition in preventing infections of orthopedic knees and hips. As a supplement to IGERT, he applied for and participated in the NSF I-Corps program, which encouraged customer-driven business model development based on Osterwalder's business model canvas [73]. Through I-Corps, this student pivoted and modified the value proposition of his research to Foley urinary catheters applications as the highest market need and the best product-market fit. After realizing the potential societal benefits, and with encouragement from outside speakers and faculty within IGERT program, this student co-founded a multidisciplinary company, AMProtection LLC with another IGERT student. This business is built on the

core competency established during their STEM Ph.D. research. Before the program, neither student considered themselves as "innovative" during an initial intake survey. This thinking was changed due in part by the development of an innovative mindset during strategic programing set forth by IGERT (**Figure 1**).

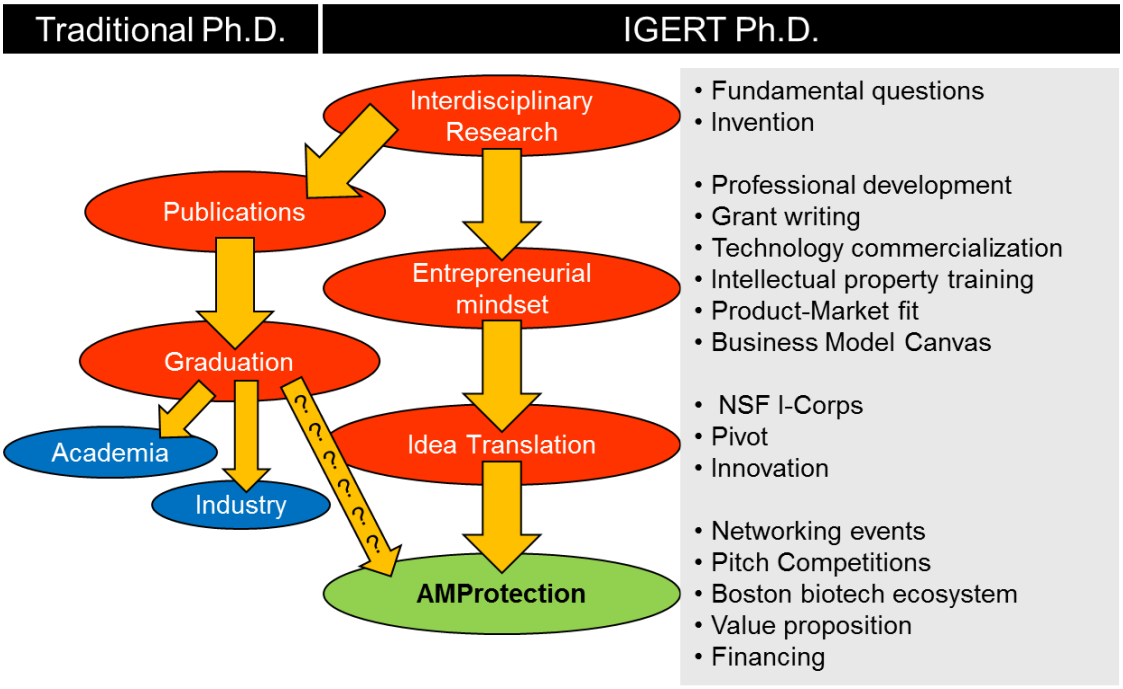


Figure 1: Ph.D. pathway at WPI with or without IGERT training. The path toward starting a new venture (such as AMProtection) is not as clear in traditional Ph.D. programs.

A difficult task is the evaluation of entrepreneurship tracks, courses and experiences due to their highly multidisciplinary, open-ended and experiential nature. Further, the acceptance of failure is a serious risk of owning a venture. Qualitative as well as quantitative evaluation is required due to variation in class sizes, high degree of individual specialization, vast array of potential career paths, and development of difficult-to-measure cognitive skills. At WPI, the IGERT faculty administered surveys comprised of closed- and open-ended questions and conducted interviews to collect data from the trainees about their experiences in the professional development (PD) courses or seminars. There were also 90-minute focus groups of 3- to 5-students, formal end-of-program reviews, and assessments based on outputs such as journal articles, internships, awards, and patents that were used to evaluate the effects of the program on the graduates.

The evaluations at WPI revealed several benefits of the IGERT education, regardless of the career path chosen by the trainees (measurable outcomes, Fig. 2). One student stated that the continuous professional development offered by the IGERT program led to several advantages over their non-IGERT peers. Other trainees agreed with this, noting that they received more training and information about how to be successful academic researchers and entrepreneurs, including knowledge about how to present their work to multiple audiences, how to apply for patents, how to prepare research for publication and, as one trainee stated, how to be a successful Ph.D. student. Some students were seen by peers as resourceful leaders in their departments because of their professional development training.

In 5 years…

- 14 finalists in university-wide poster competition (out of 800 participants)
- 5 winners, 1 honorable mention in university-wide pitch competition
- 61 conference presentations
- 21 journal publications
- 1 book
- 1 book chapter
- 6 patents or patent applications
- 1 technology license
- 2 National Academy of Inventors Members
- 1 American Heart Association Fellowship
- 1 NSF GRFP fellowship, 2 honorable mentions
- 1 VentureWell E-Team Stages 1-3 Grant
- 1 NSF I-Corps Grant
- 2 innovation awards (Kalenian and Hitchcock)
- 1 startup company
- 7 industrial and 1 government internships
- 12 international research experiences
- 7 completed dissertations (4 industry, 1 post-doctoral position, 1 startup co-founder, 1 university staff)

Figure 2: The measured outcomes of the 20 Ph.D. students in the IGERT program, spanning disciplines of Biomedical Engineering, Biology, Chemical Engineering, and Robotics Engineering.

6.6.3 What positive institutional impacts might be derived from introducing entrepreneurship into STEM education programs?

One of the most important administrative factors facilitating the success of the IGERT program has been encouragement of faculty to engage in interdisciplinary research and education. A number of the faculty participating in the program were co-instructors in courses. At many universities, there are disincentives for co-teaching courses, typically associated with the allocation of workloads. At WPI, co-instructors each fully committed to the courses and were recognized by receiving full credit of their time for their course loads. There was also support for faculty to become more engaged in the teaching of entrepreneurship through financial support for attendance at conferences in the field without the typical requirement of presenting a paper. Annual conferences such as those held by the United States Association for Small Business and Entrepreneurship (USASBE), VentureWell and The Experiential Classroom have proved valuable for learning about entrepreneurship. In particular, The Experiential Classroom provides hands-on experiences for faculty that can be immediately transferred to their content courses.

In addition to support from the administration, further support could come from private foundations such as The Coleman Foundation and Ewing Marion Kauffman Foundation. The Coleman Foundation has provided funding since 1981 to support Professorships in Entrepreneurship, a faculty fellows program, and many other entrepreneurship education programs. Grants from foundations tend to be favorably viewed by administrations in evaluating faculty. Establishing the impact that entrepreneurial activity has on the university could result in strong justification to embrace such a culture at an institution, and could be looked at favorably by potential donors. Grants from Coleman and from the Kern Family Foundation played important roles at WPI in helping STEM faculty acquire relevant skills for incorporating entrepreneurship into their courses.

At the institutional level, it was important for business faculty to collaborate with STEM faculty in establishing entrepreneurship definitions and concepts that would be seen as relevant to the mission. By establishing such standards, students could learn entrepreneurship material in a more cohesive manner. By

taking greater ownership of the curriculum, faculty avoided philosophical disagreements about teaching business concepts or courses with an entrepreneurial emphasis.

Two common phrases used by colleges to attract students are “project-based education” and “innovation”. Similar to the EDP, projects provide an opportunity to introduce entrepreneurship to students. Research is needed to understand whether entrepreneurship topics including opportunity recognition and understanding available resources will improve the project outcomes. Innovation, a key part of entrepreneurship, is a term commonly used by many universities. While the exact definition of innovation can be debated, most would agree that it involves meeting a “need” in a novel way. Further research could help determine if commercialization is an effective means to meet the need. The inclusion of commercialization early on in an EDP may be the difference between a widely accepted innovative design and one that never makes it out of the design lab. Additional studies can evaluate whether this approach is a promising way for engineering departments to include entrepreneurship into their curricula for the benefit of students and faculty.

Students and faculty in the IGERT program responded that most other graduate students would benefit from the IGERT model because it challenges students to consider the broader context of their research and teaches them to adopt professional practices early in their academic careers. Early efforts of this at WPI were implemented by the Dean of Graduate Studies via Student Training and Readiness Session (STARS) seminars. Multiple elements of the program were then disseminated through the entire university for all Master’s and Ph.D. students as part of a new Center for Graduate Student Professional Development, including a new Ph.D. Plan (Fig. 3), where IGERT-inspired principles supplement conventional Ph.D. curricula.

Figure 3: The aspects of the IGERT program that were incorporated into the full Ph.D. Plan at WPI compared to the aspects and skills that are typically acquired during conventional Ph.D. programs without entrepreneurship and innovation training.

6.7 Conclusions

Many STEM students will own and operate their own technology ventures at some point during their career, and students are requesting more entrepreneurship education [42]. A growing body of evidence shows that entrepreneurship programs add value to students and to their institutions [56, 74]. Entrepreneurship education provides students with the business creation tools they need, and can be transformative, providing significant value to the traditional curriculum. Teaching entrepreneurship concepts in STEM courses may also prove valuable to students in traditional business majors providing them with scientific knowledge pertaining to the type of company they may ultimately be running. For the university, emphasizing entrepreneurship may serve as a recruitment tool for science students, offering students a better opportunity

to obtain employment upon graduation and broaden their career choices [42, 57]. University administrators can consider entrepreneurship as a means to produce alternative career paths for their graduates, increasing their opportunities for self-employment and business ownership [24].

For WPI, the IGERT Ph.D. program resulted in invention, innovation and venture creation through the STEM disciplines. It contributed to institutional transformation as innovation and entrepreneurship become central to the university mission. Additionally, we offer this case study as an outcome of the goal of NSF's IGERT program to introduce innovative, interdisciplinary training.

This work was published in **Alexander, T.E.**, Lozeau, Linsday D., Camesano, Terri A., Hoy, Frank, *Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program.* Portland International Center for Management of Engineering and Technology (PICMET), 2019.

References

1. Bosman, L. and S. Fernhaber, *Defining the Entrepreneurial Mindset*, in *Teaching the Entrepreneurial Mindset to Engineers*. 2018, Springer. p. 7-14.
2. McGrath, R.G. and I.C. MacMillan, *The entrepreneurial mindset: Strategies for continuously creating opportunity in an age of uncertainty*. Vol. 284. 2000: Harvard Business Press.
3. Schumpeter, J.A., *Schumpeter, J.A., 1934. The Theory of Economic Development: An Inquiry into Profits, Capital, Credit, Interest, and the Business Cycle*. 1934, Cambridge, MA: Harvard University Press.
4. Schumpeter, J., *The theory of economic development. Harvard Economic Studies. Vol. XLVI*. 1911, Cambridge, MA: Harvard University Press.
5. Piperopoulos, P. and D. Dimov, *Burst Bubbles or Build Steam? Entrepreneurship Education, Entrepreneurial Self-Efficacy, and Entrepreneurial Intentions.* Journal of Small Business Management, 2015. **53**(4): p. 970-985.

6. Åstebro, T., N. Bazzazian, and S. Braguinsky, *Startups by recent university graduates and their faculty: Implications for university entrepreneurship policy.* Research Policy, 2012. **41**(4): p. 663-677.
7. Bird, B., *Toward a theory of entrepreneurial competency*, in *Seminal Ideas for the Next Twenty-Five Years of Advances*. 2019, Emerald Publishing Limited. p. 115-131.
8. Ries, E., *The lean startup methodology.* The Lean Startup, 2012.
9. Osterwalder, A. and Y. Pigneur, *Business model generation: a handbook for visionaries, game changers, and challengers*. 2010: John Wiley & Sons.
10. Blank, S. and B. Dorf, *The startup owner's manual: The step-by-step guide for building a great company*. 2012: BookBaby.
11. Blank, S., *What's A Startup? First Principles.* 2010: https://steveblank.com/.
12. Alexander, T.E., Lozeau, Linsday D., Camesano, Terri A., Hoy, Frank, *Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program.* Portland International Center for Management of Engineering and Technology (PICMET), 2019.
13. Morris, M.H., D.F. Kuratko, and J.R. Cornwall, *Entrepreneurship programs and the modern university*. 2013: Edward Elgar Publishing.
14. Morris, M.H. and E. Liguori, *Annals of Entrepreneurship Education and Pedagogy–2016*. 2016: Edward Elgar Publishing.
15. Welsh, D., *Creative cross-disciplinary entrepreneurship: A practical guide for a Campus-wide program*. 2014: Springer.
16. Mialaret, G., *Les Sciences de L'Éducation, 10th ed.* 2005, Paris: Presses universitaires de France.
17. Gunderson, S., R. Jones, and K. Scanland, *The Jobs Revolution: Chanign How America Works.* Copy Writers Incorporated, 2004.
18. Stewart, G., *Write a Grant Proposal, Start a Company, Create your own Job.* 2013.

19. Charney, A., Libecap, G., *Impact of Entrepreneurship Education.* Insights: A Kauffman Research Series, 2000.

20. Blais, R.A., *Technological Entrepreneurship and Engineering in Canada.* Ottowa: Canadian Academy of Engineering, 1997.

21. Hood, J.N. and J.E. Young, *Entrepreneurship's Requisite Areas of Development: A Survey of Top Executives in Successful Entrepreneurial Firms.* Journal of Business Venturing, 1993. **8**(2): p. 115-135.

22. Wheeler, P.A., *Educating Entrepreneurs: Have we Been Doing it Without Really Trying?* Frontiers of Entrepreneurship Research 1993 Edition, 1993: p. 644-645.

23. Kolvereid, L. and O. Moen, *Entrepreneurship Among Business Graduates: Does a Major in Entrepreneurship Make a Difference?* Journal of European Industrial Training, 1997. **21**(4): p. 154-160.

24. Menzies, T.V. and J.C. Paradi, *Encouraging Technology-Based Ventures: Entrepreneurship Education and Engineering Graduates.* New England Journal of Entrepreneurship, 2002. **5**(2): p. 57-64.

25. AUTM, *2017 US Licensing Activity Survey*, AUTM, Editor. 2017, AUTM: https://autm.net/.

26. Milligan, M.K.J. *A Message from our CEO*. 2017 [cited 2019; Available from: https://www.abet.org/a-message-from-our-ceo/.

27. Foundation, N.S. *NSF Innovation Corps*. [cited 2019 January 3]; Available from: https://www.nsf.gov/news/special_reports/i-corps/.

28. Drago, W.A. and J. Vanevenhoven, *The structure and scope of entrepreneurship programs in higher education around the world*, in *Entrepreneurial Learning*. 2015, Routledge. p. 117-133.

29. Menzies, T.V., *Entrepreneurship education at universities across Canada.* The dynamics of learning entrepreneurship in a cross-cultural university context, Hämeenlinna, FI: University of Tampere, Research Centre for Vocational and Professional Education, 2005: p. 286-309.

30. Menzies, T.V. and Y. Gasse, *Entrepreneurship and the Canadian universities: report of a national study of entrepreneurship education*. 2004: Faculty of Business, Brock University.

31. Vesper, K.H. and W.B. Gartner, *University Entrepreneurship Programmes.* Lloyd Greif Center for Entrepreneurial Studies, 1999.

32. Morelix, A., *The Evolution of Entrepreneurship on College Campuses*, in *Kauffman Foundation*. 2015.

33. Menzies, T.V. *21st Century Pragmatism: Universities and Entrepreneurship Education and Development*. in *ICSB World Conference*. 2003. Belfast, Northern Ireland.

34. National Science Foundation *Innovation Corps - National Innovation Network Teams*.

35. Duval-Couetil, N., A. Shartrand, and T. Reed, *The Role of Entrepreneurship Program Models and Experiential Activities on Engineering Student Outcomes.* Advances in Engineering Education, 2016. **5**(1): p. n1.

36. Robinson, L., *I-Corps and the Business of Great Science.* JOM, 2012. **64**(10): p. 1132.

37. Jones, C. and J. English, *A Contemporary Approach to Entrepreneurship Education.* Emerald Education and Training, 2004. **46**(8): p. 416-423.

38. Duening, T.N., *Five Minds for the Entrepreneurial Future: Cognitive Skill as the Intellectual Foundation for the Next Generation Entrepreneurial Leadership.* Journal of Entrepreneurship, 2010. **19**(1): p. 1-22.

39. Mitchell, R.K., et al., *Entrepreneurial Cognition Theory: Rethinking the People Side of Entrepreneurship Research.* Entrepreneurship Theory & Practice, 2002. **27**(2): p. 93-104.

40. Turner, T. and P. Gianiodis, *Why Entrepreneurship Programs for Engineers Fail*, in *Harvard Business Review*. 2018.

41. Fiet, J.O., *The Theoretical Side of Teaching Entrepreneurship.* Journal of Business Venturing, 2000a. **16**: p. 1-24.

42. Bryers, T., T. Seelig, S. Sheppard and P. Weilersein, *Entrepreneurship: Its Role in Engineering Education.* The Bridge, 2013. **43**(2): p. 35-40.

43. Zappe, S., et al., *Teaching to Innovate: Beliefs and Perceptions of Instructors who Teach Entrepreneurship to Engineering Students.* International Journal of Entrepreneurship Education, 2013. **29**(1): p. 45-62.

44. Brooks, R., W. S. Green, R. G. Hubbard, D. Jain, L. Katehi, G. McLendon, J. Plummer and M. Roomkin, *Entrepreneurship in American Higher Education*. 2007, Kauffman Pannel on Enrepreneurship Curriculum in Higher Education.

45. Brown, C., *Teaching New Dogs New Tricks: The Rise of Entrepreneurship Education in Graduate Schools of Business.* Kansas Digest, 1999. **99**(2).

46. Brush, C., et al., *Doctoral Education in the Feild of Entrepreneurship.* Journal of Management, 2003. **29**(3): p. 309-331.

47. Heriot, K.C., L. Simpson, and H. Stephenson, *Using adjunct faculty to teach entrepreneurship at an AACSB-accredited business program: synergies and opportunities.* Small Business Institute Journal, 2012. **8**(1): p. 47-60.

48. Fiet, J.O., *The Pedagogical Side of Entrepreneurship Theory.* Journal of Business Venturing, 2000b. **16**: p. 101-117.

49. KEEN. Available from: https://engineeringunleashed.com/.

50. Gibb, A., *In Pursuit of a New 'Enterprise' and 'Entrepreneurship' Paradigm for Learning: Creative Destruction, New Values, New Ways of Doing Things and New Combinations of Knowledge.* International Journal of Management Reviews, 2002. **4**(3): p. 233-269.

51. Hayward, G., *Evaluating Entrepreneurship in Scottish Universities.* University of Oxford Education Department, 2000.

52. Pontius, J.L. and S.R. Harper, *Principles fof Good Practice in Graduate and Professional Student Engagement.* New Directions for Student Services, 2006. **115**: p. 47-58.

53. Fayolle, A. and B. Gailly, *The impact of entrepreneurship education on entrepreneurial attitudes and intention: Hysteresis and persistence.* Journal of Small Business Management, 2015. **53**(1): p. 75-93.

54. Maresch, D., et al., *The impact of entrepreneurship education on the entrepreneurial intention of students in science and engineering versus business studies university programs.* Technological forecasting and social change, 2016. **104**: p. 172-179.

55. Ries, E., *The lean startup: How today's entrepreneurs use continuous innovation to create radically successful businesses*. 2011: Crown Books.

56. Thursby, M.C., A.W. Fuller, and J. Thursby, *An Integrated Approach to Educating Professionals for Careers in Innovation.* Academy of Management Learning & Education, 2009. **8**(3): p. 389-405.

57. Duval-Couetil, N., T. Reed-Rhoads, and S. Haghighi, *Engineering Students and Entrepreneurship Education: Involvement, Attitudes, and Outcomes.* International Journal of Engineering Education, 2012. **28**(2): p. 425-435.

58. Worcester Polytechnic Institute. *Integrative graduate education & research traineeship (IGERT)*. [cited 2019 January 3]; Available from: https://www.wpi.edu/academics/graduate/funding-opportunities/traineeship.

59. Baptista, R. and A. Naia, *Entrepreneurship education: A selective examination of the literature.* Foundations and Trends® in Entrepreneurship, 2015. **11**(5): p. 337-426.

60. Fayolle, A., B. Gailly, and N. Lassas-Clerc, *Assessing the impact of entrepreneurship education programmes: a new methodology.* Journal of European industrial training, 2006. **30**(9): p. 701-720.

61. TERC. *IGERT: integrative graduate education and research traineeship.* 2018 [cited 2018 January 3]; Available from: http://www.igert.org/.

62. Gamse, B.C., L.L. Espinosa, and R. Roy, *Essential Competencies for Interdisciplinary Graduate Training in IGERT: Final Report. GS-10F-0086K.* Abt Associates, 2013.

63. IGERT. *IGERT program document*. [cited 2019 January 3]; Available from: http://www.igert.org/library/type/IGERT-program-document.html.

64. Worcester Polytechnic Institute. *Solving real-world problems through innovative research*. [cited 2019 January 4]; Available from: https://www.wpi.edu/academics/graduate/funding-opportunities/traineeship/education-training.

65. Foundation, N.S. *NSF project reporting document*. [cited 2019 January 5]; Available from: https://www.research.gov/common/attachment/Desktop/NSF%20Project%20Report%20Template.pdf.

66. Vanderbilt University Center for Teaching. *Evidence-Based STEM Teaching: Free Online Courses Launch September 19th*. 2016 [cited 2018; Available from: https://cft.vanderbilt.edu/2016/09/evidence-based-stem-teaching-free-online-courses-launch-september-19th/.

67. The Lemelson Foundation. *The Lemelson Foundation*. [cited 2018; Available from: https://www.lemelson.org/.

68. Saint Louis University. *Saint Louis University*. Available from: https://www.slu.edu/parks/current-students/monthly-innovation-challenge.php.

69. VentureWell. *OPEN invent the future of innovation & entrepreneurship education*. 2018; Available from: https://venturewell.org/open/.

70. Georgia Tech Biomedical Engineering. *Master of Biomedical Innovation and Development (MBID)*. [cited 2018; Available from: http://mbid.bme.gatech.edu/.

71. Goldsby, M.G., et al., *Design-Centered Entrepreneurship: A Four Stage Iterative Process for Opportunity Development.* Journal of Small Business & Entrepreneurship, 2017. **29**(6): p. 477-490.

72. Stauffer, D., *Thinking Clockwise: A field Guide for the Innovative Leader*. 2005, Minneapolis, MN: MinneApplePress.

73. Osterwalder, A. and Y. Pigneur, *Business Model Generation a Handbook for Visionaries, Game Changers, and Challengers*. 2013, New York: Wiley & Sons.

74. Ohland, M.W., et al., *The effect of an entrepreneurship program on GPA and retention.* Journal of Engineering Education, 2004. **93**(4): p. 293-301.

Specific Aim 3: Chapter 7 Success factors and challenges of grassroots innovations: Learning from failure

Abstract

Grassroots innovation projects have the potential to generate novel, bottom-up solutions that respond to local situations, interests and values – solving the social, economic and environmental problems of marginalized communities; however, these projects can raise important challenges during their design, testing, development and implementation. Although extant studies identify some of these problems, the literature and practice lack a comprehensive diagnostic tool that can effectively predict the potential and success of grassroots initiatives. For this reason, important problems are often neglected, and failed projects are not thoroughly investigated and analyzed, which leads to missed opportunities of corrective learning. This study addresses this knowledge gap, proposing a diagnostic tool based on existing theoretical frameworks, which is then validated by investigating a failed grassroots innovation initiative, in order to identify its major shortcomings, and learn to avoid them in future projects. The findings also outline the role and importance of a socially inclusive approach for an effective deployment of grassroots initiatives, clarifying the relationship between grassroots innovation success and local community involvement.

7.1 Introduction

Grassroots innovations are community-led solutions to sustain- ability problems that encompass social, economic and environmental issues. They can offer promising new ideas and practices, but often struggle to scale up and spread beyond small market or social niches [1]. The grassroots approach is fundamentally different from the top-down policies expressed through government action, generating novel bottom-up solutions that respond to the context, interests and values of the involved communities. In contrast to the market activities developed and implemented by business organizations, grassroots initiatives operate in civil society arenas and involve committed activists experimenting with social innovations and/or using greener technologies [2]. Although

their application is potentially universal, their effect is arguable more dramatic in deprived communities that live beyond poverty line.

Grassroots initiatives and transformations are centered on the complex interaction between various categories of stakeholders: citizens, local community, activists, practitioners, academics, and representatives of the local or national government, having a strong inclusive dimension; however, the development and implementation of grassroots projects is not always successful. To break the social and economic routines responsible for local community problems, entrepreneurial ideas are often developed and inoculated within protective niches [3]. Then, after experimentation and improvement, the project is scaled-up and launched on the market, facing the competitive pressures determined by the existing socio-technical regime. Ultimately, the success of grassroots innovation depends on re- negotiating and restructuring the power relations between social, governmental, economic and market institutions [2].

Extant research has provided both a theoretical framework of the grassroots innovation process and specific accounts of successful projects [2, 4, 5]. Although these studies identify and outline some challenges related with the grassroots innovation process [2], these problems are not addressed using a comprehensive diagnostic tool that can effectively predict the potential and success of grassroots projects. For this reason, the main problems are easily neglected, and failed projects are not thoroughly investigated and analyzed, leading missed opportunities for corrective learning, or to an excessive focus on success stories determined by a propitious constellation of chance, hard work and inspiration. Yet, “We learn wisdom from failure much more than from success. We often discover what will do, by finding out what will not do; and probably he who never made a mistake never made a discovery” [6]. In this study, we address this knowledge gap, attempting to answer the following question: *How to identify and assess in a systemic way the*

factors determining the success of grassroots innovation projects? We address this question by (i) proposing a diagnostic tool based on existing theoretical frameworks; and (ii) applying it to investigating a failed grassroots innovation initiative, in order to identify its major shortcomings. Our study answers the call for further research "into the contexts, actors and processes under which niche lessons are able or unable to translate into mainstream situations" [2] (p. 598), in the context of grassroots in- novation projects.

In addition to identifying the main challenges of the analyzed grassroots project, our findings unveil the importance of a socially inclusive approach for an effective deployment and success of the grass- roots initiative, clarifying the role of local community involvement. Although grassroots and inclusive innovations are considered conceptually different in the extant literature, we argue that some of their characteristics are common or convergent; in particular, the direct participation of the main stakeholders in each phase of grassroots in- novation design and implementation is paramount for achieving project success.

7.2 Background

7.1.1Grassroots and inclusive innovations

Grassroots innovations can be defined as products, services and processes developed to address specific local challenges and opportunities, having the potential "to be turned into entrepreneurial ventures, and generate a livelihood for the innovator as well as others in the community" [7] (p. 6). Interest about grassroots innovations and their impact on sustainable development has been in- creasing among academics, policymakers and the general population.

The success of grassroots innovations is inevitably a function of stakeholders' goals [8]. Generally, the main stakeholders of grassroots innovation projects can be classified into three categories:

(i) *initiators*, such as non-profit organizations, scientists, academics, governmental representatives, etc.; (ii) *participants and facilitators*, such as local community, non-governmental or governmental organizations, financial sponsors, suppliers, producers or distributors, etc.; and (iii) *beneficiaries*, such as marginalized groups or individuals, under-developed or poor communities, unemployed people, environmental protection organizations, etc. Success may be directly assessed through the successful commercialization of a new product or service, or in- directly, considering the subsequent growth in income levels, community development, employment opportunities, alleviation or elimination of environmental problems, or other outcomes. Perhaps the most consistently desired result is sustainability, expressed as innovation's continuity and/or its long-term positive effects on local communities and their natural habitat.

Grassroots innovations are usually developed as alternatives of conventional solutions that are not suitable, technically relevant or financially affordable for the specific problems/needs of local communities. Examples of grassroots innovations include low impact self- housing developments, furniture recycling schemes, organic food co- operatives, farmers' markets, or local car clubs, etc. [9]. Grassroots innovations differ from market innovation in terms of context, driving force, niche, organizational form, and resource base (see Table 1).

The benefits of grassroots innovations can be divided into two groups: (i) intrinsic; and (ii) diffusion [2, 10-13]. Intrinsic benefits include personal, social and environ- mental benefits, such as self-esteem and confidence, training and skills, job creation, health improvement, social capital, reduced car use or increased recycling. Diffusion benefits are expressed through raising environmental awareness, increasing education and promotion quality, changing local government's policies, or developing sustainable development strategies and attitudes.

Several movements are known for developing grassroots innovations [5, 14]. Two important ones are the Honey Bee Network (HBN) of India and the Social Technologies Network (STN) of Brazil.

The HBN was established in 1989 by several academics, scientists, farmers and other stakeholders, to foster local innovation and disseminate traditional knowledge. Its aim is to help people to create original solutions for their problems and to face challenges in interaction with entrepreneurs, innovators and governmental institutions. The HBN provides assistance to local grassroots innovators for prototyping, patenting, incubating, seed funding and commercializing their products and services.

A group of academics, unions, NGOs, government representatives, activists, funding agencies and community groups established the STN in 2004. Its objective is to create relationships between technologists and local communities, promoting communities' participation in, and control of, innovation processes and outcomes distribution.

Inclusive innovations are closely related to grassroots innovations, involving ideas, products/services and processes that can improve the social and economic wellbeing of low-income, vulnerable and marginal groups through innovation in rural areas, emerging industries and small businesses [15]. These innovations can be considered from a dual perspective: as innovations provided for, or generated by, marginal groups, which corresponds to the general definition of grassroots innovation [16].

In general, inclusive innovations [17] (p. 8): (i) provide innovative solutions to/by low-income, marginal groups who cannot get the same economic and social benefits as other community members, due to economic, geographic, social and structural barriers; (ii) create opportunities for marginal groups to participate in innovation activities, obtaining and enjoying

positive outcomes; and (iii) should result in good quality and low cost products/services, affordable for low-income, marginal groups.

Despite their similarities ([14]) there are significant differences between grassroots and inclusive innovation in terms of stakeholders, technology, and implementation process; while grassroots innovation is usually developed by the group who also benefits from its outcomes, inclusive projects are concerned with missing institutions which prevent the mainstream innovations' benefits to reach the bottom of the socio-economic pyramid. This interplay between similarities and differences raises questions about the specific relation between grass- roots and inclusive innovation projects as, in some cases, local development initiatives in poor regions may combine the characteristics of both.

7.2.2 The role of protective niches

The protective niche – often considered in innovation studies – is a central concept for both grassroots and inclusive projects, shielding and nurturing path-breaking innovations, allowing the co-evolution of technology, user practices, and regulatory structures [18], and facilitating project expansion beyond the initial market niche.

The problems experienced by marginalized groups are related with their specific position in the present socio-technical regime, which, through specific regulations, institutions and routines, prevents them to access better education, public services or economic resources. To change their socio-economic status, activists, academics, and

Table 1: Differences between grassroots and market innovation (adapted from Seyfang *et al.* [2] p. 592).

Aspects	Grassroots innovation	Market innovation
Context	Social economy	Market economy
Driving force	Social need	Profit
Niche	Values are different: alternative social and cultural expressions enabled within the niche	Market rules are different: tax and subsidies temporarily shelter novelty from full market forces
Organizational form	Voluntary associations, cooperatives, informal community groups	Companies
Resource base	Grant funding, voluntary input, mutual exchanges, limited commercial activity	Income from commercial activity

government representatives work with these marginalized groups, assessing the specific challenges and opportunities that characterize their living and working conditions, and, on this basis, developing innovative/entrepreneurial ideas adapted to their specific market niche. These projects are tried and tested in protective spaces [19], and further improved to sustain the shock of competitive forces in real market settings. The innovation can eventually reach the necessary critical mass, force and momentum to survive and expand in larger and more complex markets, and in time, to modify the production, consumption and behavior of market actors. A largely adopted innovation can even modify the existing socio-technical regime, in- creasing the sustainability of the system, or improving the living standard of various marginalized groups.

7.2.3 Main challenges of grassroots innovation projects

Grassroots innovation projects encounter a number of challenges, classified by Seyfang *et al.* [2] into two distinct categories:

(1) Intrinsic challenges include attracting the right combination of skills, key participants, champions, resources and supportive con- textual factors in the inception phase; maintaining and developing the innovative project after the start-up; mitigating between output commercialization or public funding of the start-up activity; poor institutional fit with departmental-based funding regimes, resulting in difficulties to combine and fulfill the

different criteria of multiple, single-issue funders; and, finally, obtaining the right type of technology necessary for start-up survival and development. Unfortunately, statistics show that most grassroots initiatives spend 90% of their time simply surviving, and only 10% developing the activity [4, 20]. For this reason, they fail to develop robustness and resilience to market shocks such as funding cuts, key people leaving, turnover of volunteers, burnout of activists, or shifts in government policy. In addition, many grass- roots projects have no formally documented institutional learning, the resulting knowledge and experience being tacitly held by participants [2].

(2) Diffusion challenges mainly relate to scaling up the grassroots project from the protective niche stage to diffusion – and survival – in the competitive market. The specific characteristics of protective niches, which are so useful in the trial stage, may become barriers to project diffusion and adaptation [2]. The local community of interests developed around the grassroots project is based on location or ideological characteristics [21]; however, this geographical and social configuration may not re- produce exactly the open market structure, making the innovation project poorly prepared to confront competitive forces and consumer needs. Specifically focusing on scaling up challenges, an Organisation for Economic Co-operation and Development [16] report lists the main factors required to achieve a successful transition of inclusive projects from protected niches to competitive markets:

- the product/service responds to a strong consumer demand;
- the project is based on a viable business model;
- favorable regulatory conditions and experimentation with different implementation approaches;
- the support and drive of private entrepreneurial initiative; and,
- builing on, and efficiently using, existing infrastructures.

The success of grassroots projects is paramount for achieving a positive momentum in addressing and solving the social, economic and environmental problems experienced by marginalized groups. Every failed project represents a loss of time, money and human resources, demotivating the actors involved in these initiatives. Yet, despite the importance of properly preparing and developing grassroots innovations, the extant literature falls short of developing and proposing a general diagnostic method that can identify the potential problems of these projects. This situation is paradoxical considering the wealth of extant studies regarding innovation characteristics and success factors, the adoption/diffusion of innovations, or the models developed to assess relevant market forces. These innovation and market analysis frameworks can, in our opinion, significantly contribute to the overall success of grassroots innovation projects.

7.2.4 Useful theoretical paradigms for a strategic diagnostic of grassroots projects

7.2.4.1 Innovation & Creativity

Innovation is considered the backbone of organizational and market development, being essential for a sustainable development of companies, communities, and economic systems. Schumpeter [22] (p. 66) described innovation as: (i) introducing new products or services; (ii) developing new production methods that increase the outputs resulting from the same number/amount of inputs; decrease product unit costs; introduce new inputs or change the existing ones by creating new production schemes; (iii) opening new markets by creating selling opportunities in new geographical regions or economic sectors); (iv) finding new sources/suppliers of raw materials; and (v) reconfiguring sectors or industries, e.g., by creating or eliminating a monopoly.

When analyzing innovation projects, it is important to distinguish between innovation and creativity. The latter is defined as the production of ideas about products, practices, processes, or procedures that are novel and useful [23]. Sternberg [24] expanded the concept of creativity, emphasizing that is not limited to new ideas, including also the replication or adaptation of existing solutions to new organizational or market contexts [25, 26].

Although creativity and innovation are often used as synonyms, they are fundamentally different [27], innovation representing the successful implementation of new ideas [23]. In the case of grassroots innovations, it is necessary to separately assess the creative and the innovative phases of the project – the creative one involving mainly idea inception and development, while innovative phases are focused on the practical application of the new idea in a selected niche, and subsequently, on scaling up and launching the initiative on the real market. Considering the characteristics of creative ideas outlines by Amabile [28], we formulate the following proposition:

P1. To provide solutions to the addressed problems, creative ideas must be novel and useful for their specific context of application.

7.2.5 Innovation adoption/diffusion

The innovation diffusion theory ([29]) was successfully applied in a large number of projects [30]. Rogers [31] defined five main innovation characteristics that influence their adoption rate:

- Relative advantage – the additional benefit offered by the innovation in comparison with the existing offer on the market;
- Compatibility – the consistency of the innovation with adopters' values, past experiences, and needs, but also with existing regulatory and institutional frameworks;
- Complexity – the difficulty of understanding and using the innovation;

- Trialability - the capacity of targeted users to interact with the in- novation; and
- Observability - the visibility of innovation within the society at large, or in a social/professional group.

According to this model, innovation diffusion is influenced by the perceptions of potential consumers, regarding the capacity of the innovative idea/product/service/process to better satisfy their needs in their specific living and working environment. Although an objective measurement is not possible, these dimensions have been proven to accurately predict the rate of innovation adoption by the targeted population.

In the context of grassroots initiatives, we propose the application of the innovation diffusion model to assess the capacity of innovative ideas to be successfully and sustainably adopted in the protective niche that serves as testing ground for their further development. On this basis, we formulate the following proposition:

P2. To evaluate the capacity of grassroots' innovation idea to be successfully adopted and diffused in the protective niche, it is necessary to analyze its (i) relative advantage; (ii) compatibility; (iii) complexity; (iv) trialability; and (v) observability in relation to the targeted population.

7.2.6 Market forces

Scaling up and launching a grassroots innovation on the open market requires a thorough analysis of the forces that shape the competitive structure of the business environment, a useful tool being Porter's (1980) Five Forces' Model [32]. When studying the competitive environment, firms traditionally concentrated mainly on direct competitors. Porter's Five Forces model expands market analysis, including the threat of new market entrants and of substitute offers, as well as the bargaining power of customers and suppliers. The five forces of this model are:

(i) the bargaining power of suppliers: the number, size and market positioning of suppliers will

influence the profitability of the project, because the price charged by suppliers represents an important cost. When suppliers are few and/or raw materials are scarce, the project may encounter significant scaling up problems, as market success will require an important increase in production, and subsequently, in the volume of inputs;

(ii) the bargaining power of customers: launching the grassroots project outside the protective niche may modify its scope and perception, as open market customers can have different needs or purchasing power;

(iii) the intensity of direct competition: outside the protective niche, the grassroots project may be confronted with direct competitors, some having more experience, offering more value or having a better reputation than the scaled-up venture;

(iv) the threat of substitutes: the open market may include substitute offers that are easier to use or are perceived to have a better value than the grassroots project; and

(v) the threat of new entrants: the potential success of the grassroots project does not guarantee its durability, as the innovative idea may be copied and replicated by new entrant competitors; thus, the capacity to protect the grassroots innovation and quickly build a positive reputation represent critical factors for a sustainable success of the project.

In addition to the above five, we also propose to analyze the bar- gaining power of retailers/distributors that we consider as a sixth relevant force influencing market structure and competitive situation, although excluded in the initial Five Forces' Model. In many markers, independent distributors are controlling the access to, and often the perception of, new products' value, risk and utility. Taking into account these six constitutive market forces, we formulate the following proposition:

P3. To evaluate and predict the capacity of the grassroots project to be successfully scaled-up and launched on the open market, it is necessary to analyze: (i) the bargaining power of suppliers; (ii) the bargaining power of customers; (iii) the intensity of direct competition within the market; (iv) the threat of substitutes; (v) the potential threat of new market entrants, and (vi) the bargaining power of retailers/distributors.

To translate these theoretical propositions into a practical diagnostic tool, we propose a tripartite division of the grassroots project (see Table 2):

- *The inception phase* – comprising the analysis and definition of the problems, the creation of new ideas, and the assessment of idea's adequacy to address the identified problems – in other words, its contextual novelty and potential utility;
- *The protective niche phase* – the idea is developed into a prototype that is tested, developed and refined with a protective market niche, which provides favorable conditions for experimentation and learning; we contend that, in this phase, the success of the grassroots project can be predicted by assessing the five factors of the in- novation adoption/diffusion model;
- *The open market phase* – the project is scaled-up and launched in a competitive environment; to ensure its sustainable success we pro- pose the analyses of the six forces that characterize and shape market structure and functioning.

The phases, elements and the main analytical questions included in this diagnostic tool are presented in Table 2. The proposed diagnostic tool is by no means exhaustive, as, depending on the context and type of the investigated grassroots innovation project, the presented analytical questions can be modified, or other relevant questions may be added. To demonstrate the application of this diagnostic tool, our paper presents a detailed case study of a failed grassroots

innovation project, to which we apply, subsequently, the proposed diagnostic tool. The next section explains the methodology applied for data collection coding and analysis.

7.3 Methodology

To analyze and evaluate the practical implementation of a grass- roots initiative, we apply a case study methodology. Acknowledging the importance of a specific social, economic and environmental context for developing and implementing sustainable grassroots innovations, our approach is based on situated human practice. The dialectic and dynamic interplay between human agency and institutions ([33]) is investigated in other grassroots innovation studies [34-36]. Institutions exist through human expressions and practices, which are always situated in a specific context and cultural frame; at the same time, the existing socio-economic structures – representing the institutional background of the society in a specific point in time – influence and shape the situated human practices.

Table 2: Phases, elements and analytical questions included in the proposed diagnostic tool

Project phases	Diagnostic elements	Analytical questions
Inception	Idea novelty	• How the developed creative idea compares in terms of novelty with existing product/services? • Is the idea completely new, or represents a creative adaptation of an existing solution?
	Idea usefulness	• How effective could be this idea to address/solve the identified problems? • Is there any danger that the application of the new idea will create additional problems?
Protective niche	Relative advantage	• What are the additional benefits provided by the prototype in comparison with existing products/services?
	Compatibility	• What is the compatibility of the prototype with the existing values, attitudes and practices of targeted customers?
	Complexity	• What is the level of complexity of the prototype in comparison with existing product/services? • What is the perception of targeted customers regarding the complexity of the prototype?
	Trialability	• How can the prototype be tested/tried by the targeted customers and how easy is to organize and enact the trying procedure?
	Observability	• How visible is the prototype for the targeted consumers, in comparison with existing products/services? • How can this visibility be improved?
Open market	Customers	• What is the socio-economic profile, the needs and the behaviors of targeted customers? • What is the bargaining and purchasing power of targeted customers regarding the new offer?
	Suppliers	• What is the number, profile and availability of organizations supplying raw materials? • What is the quality of their offer and services? • What is the bargaining power of the existing suppliers?
	Distributors	• What is the number, profile and availability of organizations distributing the product/providing the service? • What is the quality of their offer and services? • What is the bargaining power of distributors?
	Direct competitors	• What is the number, profile and market reputation of organizations offering similar products/services? • What is the market positioning of their offer? • What is their market/competitive power?
	Products of substitution	• What existing products/services can satisfy the same customer needs and who is offering them? • What is targeted customers' perception regarding these products/services? • What is the projected demand elasticity of our product/service in relation to a price change of the products/services od substitution?
	Potential entrants	• What is the number, profile and market reputation of organizations that can potentially develop and offer similar products/services? • What is their market/competitive power and positioning?

Our methodological choice is justified by the necessity to under- stand the complex interdependence between social, economic and environmental factors in grassroots innovation

projects. Instead of using a hypothetical-deductive method to identify, measure and validate the relationship between quantitative variables, we adopt a holistic-inductive qualitative approach based on representative case studies [37]. Case study analysis is particularly adapted to investigate of complex socio-economic phenomena, because of its capacity to combine primary and secondary data, and to integrate quantitative and qualitative elements into a complex narrative. Case study methodology was previously applied for investigating the success factors of grassroots innovation projects [4, 20, 38-41]. In addition, case studies are often used in exploratory research that tries to answer "why" or "how" questions as they "retain the holistic and meaningful characteristics of real-life events" [42].

The choice of the case study analyzed in this paper is not random, as it provides detailed insights into the challenges, phases, actions, and consequences of a grassroots innovation project that attempts to solve social, economic, and environmental problems experienced by marginalized groups. To select this case study, we applied a multi-stage process of secondary data collection, processing and analysis. After realizing an extensive literature review regarding grassroots and inclusive innovations, we analyzed a series of grassroots initiatives launched and coordinated by Men on the Side of the Road (MSR), a Namibian non-profit organization that works to improve the living conditions of marginalized groups in informal settlements. The archival data and the detailed description of these MSR initiatives were accessed using media publications and Worcester Polytechnic Institute database of student projects.

Namibia gained independence from the Republic of South Africa in 1990. The last colony in sub-Saharan Africa to become independent, it was the first to specifically base its future on the small business sector [43]. After years of apartheid, its economy was fragile and, despite the government's attempts to provide basic services ([44]) and develop the economy [43], poverty

and unemployment remained extremely high. In 2016, the official un- employment rate in Namibia reached 34%, rising from 28.1% in 2014 [45]; however, these statistics did not consider the partial unemployment of a large number of day laborers, while many women who took care of their households were not included in the counting [46].

Poverty and unemployment is endemic especially in rural areas, where people try to live from subsistence farming [47]. Many of these people migrate to urban areas in search for jobs and a better living – but this solves little, given that the existing infrastructure cannot accommodate a sudden increase in urban population. Many migrants end up living in informal settlements, located at the periphery of large cities [48], and becoming socially marginalized groups.

Prompted by its representativeness and relevance for grassroots innovation research, we selected to study the Paper Block project developed and implemented in the Katutura settlement – located at the outskirts of the Namibian capital, Windhoek. We used only secondary sources of information, mainly from the materials published by the Man on the Side of the Road association, and material provided by the Worcester Polytechnic Institute.

7.3.1 Data coding

We manually coded the collected data on the main themes associated with our research objectives – stages of project deployment, main participants and stakeholders, implemented actions, their con- sequences, and the main encountered challenges. The process of data coding and analysis included three main phases:

First, the collected information was divided into units of text [49]: parts of sentences, complete sentences, or groups of sentences related to the same theme, which were classified into clearly defined categories. The size of these units depended on [50]: (i) the research relevance of the selected unit, and (ii) its interpretability without additional information. To reduce interpretation

bias, this phase was realized in parallel by two researchers, who separately identified and classified the units of text into coded categories. Then, the two researchers confronted their work and, together with the other co-authors, consensually decided a final coding classification. Second, after identifying and classifying the units of texts, we analyzed their meaning taking into account and relating each project stage with the assessment variables included in the proposed diagnostic tool. Third, we synthesized the findings, integrating and logically-ordering various elements of the investigated grassroots project, by connecting the main processes with the influencing/facilitating factors and analyzing the embeddedness of this initiative into the local social, cultural, economic and environmental context. The next section presents the selected case study.

7.4 Case study: The Paper Block Project in the Informal Katutura Settlement

7.4.1 The problems

Coupled with endemic unemployment, most families in the informal Katutura settlement lack access to basic services, such as water or electricity. Cooking and heating is mainly performed using paraffin or wood [51]; however, these fuels present important problems. Paraffin is highly effective in terms of caloric power, but is dangerous to transport, store and use. Paraffin lamps or stoves can start fires that spread quickly given the density of shacks in the informal settlement. On the other hand, firewood is growing scarcer and more expensive. Women often walk several kilometers to find scrap wood, and many farmers are now forbidding people to collect wood on their property, as in some cases live branches or entire trees were cut and stolen. Other people are using wood from discarded pallets or boxes or buy it from vendors at a price of 10 Namibian dollars (N$10) for 5 to 7 wood pieces – an unaffordable price for many very poor families [52].

Finally, the increasing demand of wood has created many environmental problems, such as deforestation, soil erosion, unbalance of the local water cycle, loss of habitats for many species,

and, most seriously, desertification. Although woodcutting is strictly forbidden without a license, the local government could not stop all abuses. As Namibia has the driest climate in the sub-Sahara, deforestation can easily lead to loss of fertile soil through desertification.

7.4.2 The initial project

The Paper Block grassroots project was initiated in 2011, by Men on the Side of the Road (MSR) – a South African non-profit organization which expanded to Namibia in 2007 [53]. MSR's main mission was the inception, organization and implementation of grass- roots projects involving local residents, communities, non-profit organizations, voluntary social workers, and government representatives, to enable marginalized people to acquire skills and attitudes leading to long-term or self-employment. The organization "encourages in- dependence and responsibility and gives purpose and a strong sense of self-respect" (http://msr.org.na/about-us/who-we-are-and-what-we- do/). After the failure of the initial Paper Block project, a group of students from the Worcester Polytechnic Institute, in Massachusetts, attempted to revitalize and relaunch the initiative.

The Paper Block project addressed a series of inter-related problems, centered around the increasing wood scarcity for cooking in Katatura: *first,* to provide an effective alternative to the existing fuels used for cooking; *second*, to develop an sustainable business model involving local community members – as suppliers, producers, distributors, promoters and consumers of the alternative fuel, resulting in increased levels of income, self-employment, acquisition of skills and attitudes leading to entrepreneurial initiatives, stronger community relation- ships, and a better quality of life; and *third*, to alleviate the environ- mental problems determined and aggravated by wood scarcity.

The key success metrics for MSR were thus: (i) the successful development and launch of an alternative type of fuel; (ii) the sustainable implementation of an economically-viable business model centered around the production, promotion, distribution and consumption of the new fuel; subsequently leading to (iii) the acquisition and application of entrepreneurial skills and attitudes; (iv) job and entrepreneurial opportunities; (v) a better level of life quality and environmental protection. However, the strong qualitative and interpretative dimension of these metrics does not permit a clear quantification of results.

Using a United Nations Development Program Grant, MSR worked together with local families, community leaders, and businesses to develop an alternative to firewood that could be manufactured from waste products already available in the city [54]. After in- formal discussions with Katatura residents, service providers, and community leaders, the idea of a compact paper block gradually emerged as a potential alternative fuel. The block was made from a mixture of paper, sawdust, and water. By collecting waste office paper from a waste management organization, and sawdust from a Vocational Training Center, MSR members were able to produce a paper block with almost no supply cost – as raw materials were free, only their transport to the workshop represented a cost.

After producing 2000 paper blocks, the MSR employees attempted to advertise and sell them in Katutura. However, the means employed were highly ineffective. To promote the paper block, MSR produced a brochure to explain product benefits, and used word-of-mouth methods. To commercialize the paper block, the head of the project decided to personally try door-to-door selling. He was only able to take approximately 40 blocks at a time into the community, which he tried to sell for N$1 each. After paying the taxi fare of N$18 to transport the paper blocks from the MSR office to Katutura, and giving to MSR 50% of earnings – to pay the activists

producing the blocks, he was left with little remuneration for his efforts On the other hand, his constant and unpredictable mobility within the informal settlement made difficult for repeat customers to find him. The project was stopped in 2012, as the commercial adoption of the paper block in Katutura failed to materialize.

7.4.3 Redesigning the product and improving the project

In 2014, the Paper Block project was analyzed and re-launched by a group of students from Worcester Polytechnic Institute, this activity counting as an Interactive Qualifying Project for their Bachelor of Science degree. The group worked together with the MSR employees and local citizens to understand the causes of the previous failure, and, on this basis, to develop and test a new paper block in terms of both composition and design. Finally, after assessing the existing methods to advertise and commercialize the product, they made a series of re- commendations concerning the scaling up of the project.

Although the first project was deemed a failure, the Katutura community was still perceived as a protective niche viable for a follow-up effort. Informal settlements such as Katutura tend to be economically and socially insular. Trust based on close, long-term interpersonal relationships between local families, service providers, and local enterprises, represents an important factor when attempting to deliver a new product or solution. In Katatura, there was no direct or indirect competition for the Paper Block project, as all recycled paper and sawdust suppliers worked exclusively with the MSR organization, and the existing commercial infrastructure was suited for such an initiative. On the other hand, the similarity of Katutura with other informal settlements sprawled around the capital city, and the marginalized status of its residents, provided ideal conditions for experimenting a product that could be subsequently launched in other poor areas. Thus, Katatura included the two defining characteristics of protective niches ([19]) -

nurturing (as its ecosystem included all the elements facilitating the introduction of an alternative type of fuel) and shielding (as various competitive market forces had a low impact on the development and viability of the project).

A thorough analysis of the initial project unveiled its main short- comings. First, the number of directly involved MSR members and employees was very small – only eight people providing data or re- commendations regarding the further development of the project. Second, the project was not sufficiently developed and tested within the local community before attempting a commercial launch. Third, the initial paper block had a poor caloric efficiency, producing too much smoke and ash, but insufficient heat, which discouraged potential customers.

To improve the product, the students' team repeatedly interacted with Katutura's inhabitants to understand their cooking routines. They discussed with 25 residents, primarily women, observing how they cook their meals, while also enquiring about the advantages and shortcoming of various types of fuels.

The residents usually cook two meals a day – lunch and dinner. The alternatives to firewood include stoves with paraffin or propane gas, but they cannot be used to cook meat because it takes too long and costs too much. Both paraffin and gas stoves have a high starting cost, as the stoves are sold for approximately N$100. A three-kilogram tank of gas is typically sold for N$70 and lasts approximately a month if used sparingly and in addition to wood. On average, a liter of paraffin costs N$15 and can cook three light meals. By comparison, N$10 worth of wood allows a family to cook one or two substantial meals such as meat and corn. Although the cost per meal is more expensive, wood fire can be used for foods that require more cooking heat and time.

The students distributed paper blocks to seven families from Katutura to test the initial product and, a week later, they collected direct feedback from five of them. All participants reported

success in using the block as a fire starter, but they were not satisfied by its heating power, as most people needed to add firewood to complete their cooking.

The team tested several alternative compositions and shapes to improve the performance of the paper block, measuring the time taken to reach maximum temperature, the maximum change in the delivered temperature, and the change in temperature per minute. Comparing these indicators, the students found that the block composed of two parts paper to one part sawdust performed the best, while the one with two parts paper to one part sawdust and sticks was the worst. Finally, the blocks – made of two parts sawdust to one part paper – were too fragile, falling apart during the molding process. The team also developed a new paper block design with two parallel holes running lengthwise through the block center. The new block was thinner and less compressed than the original one, producing more heat - due to increased surface to volume ratio and an improvement of the airflow through the block – and being comparable with the energy released by firewood.

7.4.4 Recommendations for scaling up the project

7.4.4.1 Product

The students' team found and tested a composition of the paper block that allowed not only the manufacturing of improved products, but also the recycling of the existing unsold stock. In a real test made by a resident woman who was cooking a traditional thick maize porridge, six recycled blocks were used for a cooking time of approximately 15 min, but the remaining ashes continued to produce a significant amount of heat for another ten minutes.

7.4.4.2 Price

Following a study of the existing fuel market, the students' team suggested a N$1.5 retail price for the redesigned paper block, 50% higher than the initial price: “From our findings, people who cook for their families spent approximately N$10 per day while those who cook more commercially spent approximately N$10 per meal. We estimated that a meal can be cooked using

four to five blocks, therefore the blocks should be able to compete at a retail price of N$1.50 each, as cooking one meal would cost approximately N$6-7.50." ([55], p. 50).

7.4.4.3 Promotion

The team also studied various ways in which commercial news spread in informal settlements. Many respondents indicated that the best methods to promote paper blocks would be regular community meetings, or radio advertisements on Namibia Broadcasting Cooperation (NBC) Oshiwambo Radio, as many people use this media channel for their daily news and entertainment.

7.4.4.4 Distribution

One of the major drawbacks of the initial project was the lack of a consistent and convenient selling location. The team suggested involving local retailers, as people will buy paper blocks when acquiring food or other merchandise for their daily living. In addition, as retailers will replenish their inventory from the MSR, the time previously spent to sell the blocks, could be used by MSR members for production. Through market research and discussions with residents, the team identified three vendors willing to add the paper blocks to their existing stock: a shop offering fruits, vegetables and small household goods, the Kaperona Trading Enterprises specialized in furniture and firewood, and a small shop selling snacks and repairing shoes.

7.4.4.5 The business model

The students' team suggested that MSR workers should only deal with paper blocks production; using the MSR office, they could make around 100 blocks in eight hours. The retailers will then come to the MSR office and purchase the blocks for N$1. MSR will collect the money and keep N$0.10 per block for the overhead, which covered equipment maintenance and material transportation costs; the remaining balance being paid to those who made the blocks. Then, retailers could sell the blocks for a recommended price of N$1.50 or higher.

The student team observed that a N$8 stipend per day paid was sufficient to retain the workers producing paper blocks. Unfortunately, since the project was terminated shortly afterwards, the established employment objectives were not met. Further follow-up assessments are required to determine if the skills and attitudes gained by workers resulted in self-employment, but it is reasonable to expect lag times between training and venture creation [56].

7.4.4.6 General recommendations

The team also provided two general recommendations for further developing the project:

- MSR should continue to develop and test new paper block compositions, attempting to improve its heating time and power; and
- MSR members should actively promote the paper block in Katutura social communities.

7.5 Findings

Using the stakeholders' classification proposed in the background section, we identify: the MSR members – including local voluntary activists – as initiators and participants, the United Nations Development Program Grant, the students' team, local families, business, and community leaders as participants and facilitators; and local customers, unemployed people, and the natural habitat, as potential project beneficiaries.

To assess the advantages and the shortcoming of the Paper Block project, we apply the proposed three phases' diagnostic tool; however, its direct application presents some challenges, as the protective niche phase is not clearly apparent in the initial project. On the other hand, the research and testing realized by the students' team, as well as the improvement of the initial product, qualify well as a protected niche phase, but for the scaling up and launching phase we only have some partial recommendations. To solve this problem, we will treat this grassroots project in its entirety,

distinguishing however between the first and the second wave of activities. The strategic diagnostic of the project is summarized in Table 3.

Table 3: A strategic diagnostic of the paper block project.

First wave of activities	Second wave of activities
Inception: (1) novelty and (2) usefulness	
(1) The transposition of an existing idea –alternative fuels, into a new context – the Katutura informal settlement.	(1) The idea is creatively tested and developed
(2) The idea addresses current social, economic, and environmental problems, but its implementation is fraught with challenges and shortcomings.	(2) The capacity of the idea to address the targeted social, economic, and environmental problem is improved and its application is facilitated by students' work.
Innovation adoption/diffusion: (1) relative advantage, (2) compatibility, (3) complexity, (4) trialability, (5) observability	
(1) relative advantage not existent or very low	(1) relative advantage significantly enhanced as a result of repeated tests and improvements
(2) low to medium level of compatibility with local cooking customs	(2) a better understanding of compatibility issues as a result of students' research
(3) low complexity	(3) low complexity maintained
(4) very low trialability	(4) limited trialability, but recommendations made for improvement
(5) very low observability	(5) limited observability, but recommendations made for improvement
Market forces: (1) customers, (2) suppliers, (3) distributors, (4) direct competitors, (5) products of substitution, (6) potential entrants	
(1) low bargaining power, but also very low purchasing power	(1) interested, but limited in their capability to regularly buy the product
(2) no specific problems as raw materials were free and MSR members were providing the labor	(2) raw materials freely available and cheap labor force, possible problems if the project is successfully scaled up and the demand grows
(3) low cost, but highly ineffective distribution method	(3) improved distribution method, but unpredictable evolution of price and competition for the scaled-up project
(4) non-existent	(4) non-existent
(5) firewood, wood scraps from pallets, paraffin, gas, coal	(5) firewood, wood scraps from pallets, paraffin, gas, coal
(6) non-existent	(6) non-existent but possible if the project is successful scaled-up leading to growing demand

Although the Paper Block idea was developed from the very beginning, its testing, refining and validation took place only after the re-launch of the project, these activities being initiated and realized mainly by the students' team, with the support of local MSR activists. Unfortunately, the five characteristics influencing product adoption/ diffusion have poor scores in the initial project; however, through thorough research and repeated testing, the student's team succeeded to significantly improve the relative advantage and the compatibility of the proposed product, while making insightful recommendations for enhancing its trialability and observability. Finally, the analysis of the six market forces realized by the students' team provides a better understanding of the potential challenges faced by the project in a fully-competitive market, especially concerning customers', suppliers', distributors and potential entrants' evolution in the case of product success.

It is also important to outline the lack of community implication in the initial project. This sheds a light on the possible connection between grassroots projects and inclusive innovations, as initial

project failure was probably caused by the lack of social inclusion of local residents, businesses and community leaders for the inception, testing and development of the product.

7. 6 Discussion

Grassroots innovation projects have the potential to provide creative solutions to many economic, social and environmental problems; however, for this potential to be actualized, they have to successfully transition the three phases of inception, adoption and scaling up in order to reach maturity and market sustainability. Unfortunately, the investigated Paper Block project encountered both intrinsic and diffusion challenges [2], which prevented its full development and success.

The Paper Block project initially failed because of several important shortcomings. As the applied diagnostic tool is clearly indicating, in the inception phase, the idea was sufficiently novel for its context of application, and the potential usefulness of the initiative was adequately addressing important social, economic, and environmental issues. However, the protective niche phase was almost non-existent, as the MSR members took the initiative to manufacture and sell paper blocks without developing and testing a prototype. This is clearly reflected in the extremely low level of new product's relative advantage in comparison with existing alternatives. Because it failed, we cannot consider the initial project as including a real scaling up and launching phase, although the MBR members were confronted – in a very limited way - with the demands and structures of the open market.

A general shortcoming of the initial project was the lack of involvement of the local community in developing the idea and testing the product. Although grassroots innovations have different characteristics than inclusive projects, we cannot rule out a complementary connection between the two types of initiatives. Activist groups, local government representatives or

academics, often realize idea inception in grassroots innovation, but such a project cannot be developed and tested without the direct implication of the local community. On the other hand, grassroots projects aim to improve the economic, social and/or environmental problems of marginalized groups, which is also the aim of inclusive initiatives. To achieve success, grassroots innovations require a strong inclusive dimension, local residents often re- presenting the objective, subjects, actors, context, and the testing ground of the project.

The role and importance of local community involvement is demonstrated by the difference between the approach and result of the two Paper Block projects. A significant improvement of the initial project was realized by the students' team mainly through repeated interactions with various local constituencies. This recurrent dialogue provided relevant and valid data about residents' behavior, needs, wants and customs, as well as a better understanding of local inter-personal relationships and commercial infrastructure.

After identifying the problems of the initial project, the students' team addressed the main issue – the lack of relative advantage of the new product – modifying its composition and design and augmenting its caloric power. Then, they repeatedly tested the product, using residents' feedback and their own observations to develop a viable business model, to ensure a wide diffusion and use of the block paper, with a positive financial return. In addition, the paper blocks produced in the initial project and left unsold were recycled to manufacture the new product version. However, students' involvement stopped in the protective niche phase – which was not completed because neither the promotion nor the distribution of the new paper block was fully tested. Despite their positive contribution to re-launch the Paper Block project, the students' team did not have the opportunity to analyze the six market forces, and thus their recommendations do not take into account many potential challenges related to scaling up and

launching the project on the competitive market. An analysis of the competitive environment may have indicated the following challenges regarding the long-term sustainability of the project:

- the availability and cost of raw materials may change in the future – especially if the demand and competition is developing, which could challenge the existing business model;
- the affordability of the new product was not analyzed in relation to the price and the customer segment, as it is possible that the poorest families – which initially represented a main project target – will not be capable to regularly buy paper blocks; also, the price of the paper block may rise if monopolies emerge in the market;
- the product promotion was not tested, and several potential methods – such as the word of mouth – were not fully considered; the distribution patterns are still unknown, as the product still had to be commercially launched using the selected retailers;
- the possible appearance of new entrants or followers on the alter- native fuels' market was not taken into account, neither their potential effect on the market structure, competition and demand.

Taking all these elements into account we contend that many grassroots projects need to adopt a socially-inclusive and customer- centered method of inception, testing, development and implementation (see Fig. 1). The initial creative idea must be based on a thorough analysis of the context and a clear identification of extant problems and needs of the targeted group(s). A protective niche should be defined within the local community to test and further refine the creative idea, transforming it into a viable product/service prototype. Then, based on a thorough analysis and understanding of manifest and potential market forces, the prototype must be scaled up and launched using a sustainable business model. The process, however, does not stop at this

point, as the evolution of the product/service should be closely followed, assessing customers' response, competitive changes, and the distribution of benefits within the general population.

Outcomes' assessment should take into account not only the primary objectives (such as developing, launching and commercializing a new product), but also the associated/indirect benefits (such as improved environmental protection, development and diffusion of entrepreneurial skills, or new ventures' creation) The contact with various stakeholders and constituencies would therefore continue to represent a valuable feedback source during the entire lifecycle of the product/ service, as both challenges and benefits may evolve in time.

7.7 Implications

7.7.1 Theoretical implications

Our findings indicate the potential application of a systemic diagnostic tool to assess the potential of grassroots innovation projects during idea inception, protective niche adoption, and product/service launch on the open market. Considering the most important requirements, objectives and characteristics of each of these phases: i.e., the novelty and usefulness of the creative idea, the factors facilitating the adoption/diffusion of the innovation, and the various forces determining the structure and the evolution of the market, this diagnostic tool creatively combines the creativity theory [28], with innovation adoption ([29]) and with the Five Forces Model ([32]) to provide a practical method for project analysis and evaluation.

On the other hand, our findings indicate that the success of grassroots innovation initiatives depends on the adoption of a socially-inclusive approach, in which activists, academics and/or government representatives permanently interact with the local community in every phase of project design and implementation. For this reason, we content that grassroots and inclusive innovations have common or convergent objectives and approaches, as demonstrated by the Paper Block project. On this basis, we propose the integrative concept of socially-inclusive

grassroots innovations, defined as products, services and processes developed to address specific local challenges and opportunities, whose success depends on directly involving and including the project stake- holders in each stage of innovation design, implementation and exploitation.

7.7.2 Practical implications

The analysis of the presented case study unveils a series of challenges regarding the development and implementation of grassroots projects. It is interesting to note that the main problems of the Paper Block project stem from an unsuccessful transition from protective niche phase to the open market, lacking the following success factors ([16]):

- the product/service should respond to a strong consumer demand;
- the project must be based on a viable business model;
- favorable regulatory conditions and experimentation with different implementation approaches;
- the support and drive of private entrepreneurial initiative; and, finally
- building on and efficiently using existing infrastructures.

None of these conditions were fulfilled in the initial Paper Block project, and only partially realized in the second attempt. These shortcomings finally led to project termination in both cases, approximately after one year of planning and activities.

7.8 Conclusion

Our study provides several original contributions to the grassroots innovation literature. *First*, we discuss the main characteristics of the concept, clarifying its definition and scope. *Second*, focusing on grass- roots innovation projects, our paper mobilizes three well-known theoretical models: the creativity theory ([28]), the innovation adoption/diffusion model ([29]), and the Five Forces' Model ([32]), to develop a diagnostic tool for assessing the potential of grassroots initiatives, including three successive phases: (i) idea inception; (ii) testing and developing a

prototype in a protective niche; and (iii) scaling up and launching the product/service on the open market. *Third*, we demonstrate the use of this tool by analyzing and evaluating the Paper Block project implemented in Katutura, Namibia – identifying and explaining its major shortcomings that ultimately led to failure. Finally, the analysis of this project indicates the primary role and importance of a socially inclusive approach, which directly involves the local community at every stage of project design and implementation.

This study has several limitations determined by the applied methodology. To provide the necessary details for a good under- standing of the Paper Block project, we used a case study approach, which facilitates deep analysis, but limits the general applicability of our findings. Future research should attempt a further refinement and validation of our diagnostic tool, using either case studies or an extreme event approach. However, besides its shortcomings, our paper facilitates a better understanding of the main challenges encountered in grassroots innovation projects, and indicate possible action levers for activists, academics, and/or government officials involved in these initiatives.

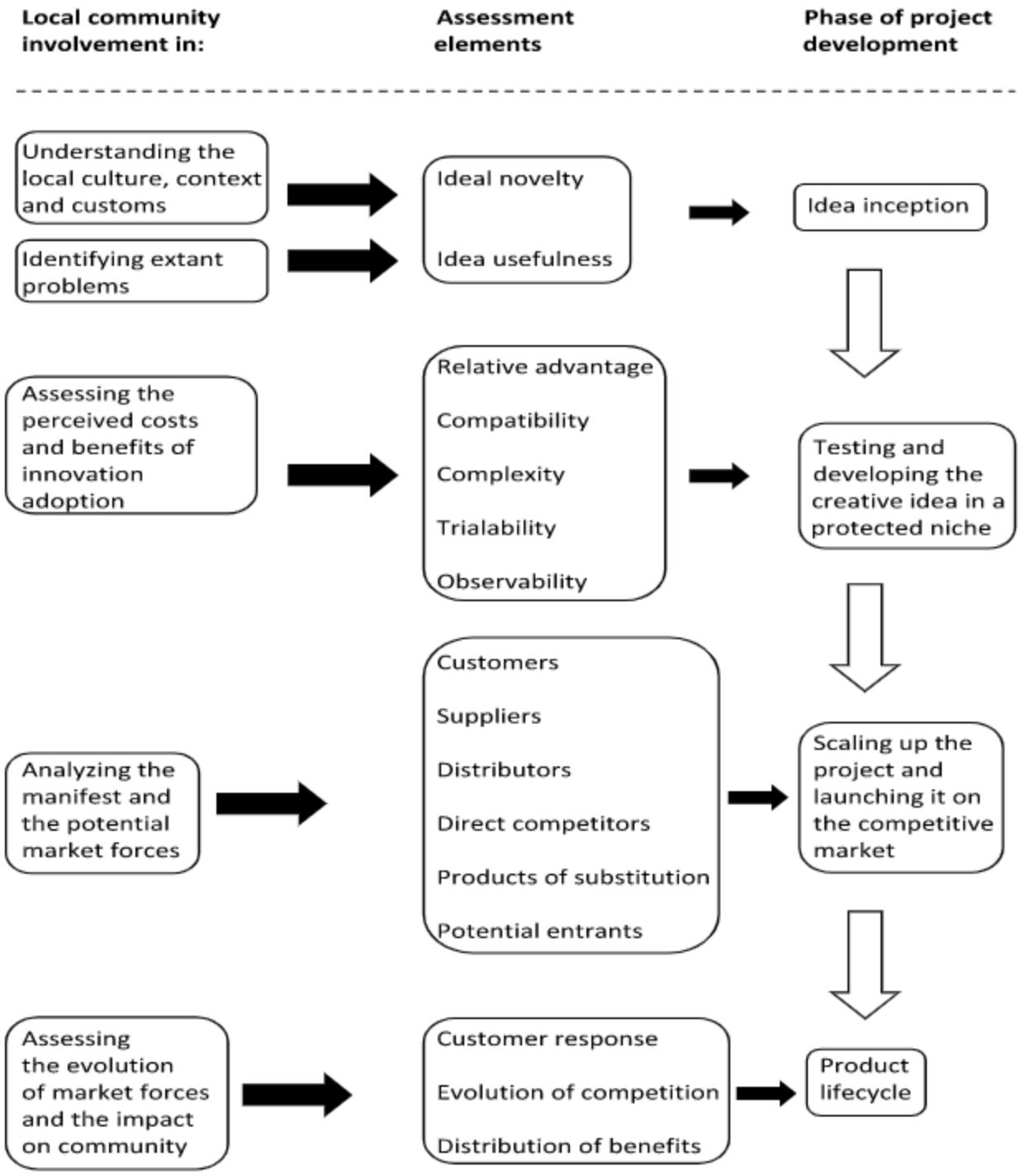


Fig. 1 The importance and role of local community involvement in inclusive grassroots Projects.

This work was published in Dana, L. P., Gurău, C., Hoy, F., Ramadani, V., & Alexander, T. (2019). Success factors and challenges of grassroots innovations: Learning from failure. *Technological Forecasting and Social Change*.

References

1. *Grassroots Innovation*. [cited 2017 September 20]; Available from: https://grassrootsinnovations.org/about/.
2. Seyfang, G. and A. Smith, *Grassroots innovations for sustainable development: Towards a new research and policy agenda.* Environmental politics, 2007. **16**(4): p. 584-603.
3. Smith, A., *Translating sustainabilities between green niches and socio-technical regimes.* Technology analysis & strategic management, 2007. **19**(4): p. 427-450.
4. Church, C., *Sustainability: The importance of grassroots initiatives.*, in *Grassroots Innovations for Sustainable Development Conference*. 2005: London.
5. Gupta, A.K., et al., *Mobilizing grassroots' technological innovations and traditional knowledge, values and institutions: articulating social and ethical capital.* Futures, 2003. **35**(9): p. 975-987.
6. Smiles, S. 2017; Available from: https://www.goodreads.com/quotes/390439-we-learn-wisdom-from-failure-much-more-than-from-success.
7. Guttikonda, A., *From innovators perspective: process of grassroots innovation in Andhra Pradesh and Telangana*. 2016, Massachusetts Institute of Technology.
8. Bal, M., et al., *Stakeholder engagement: Achieving sustainability in the construction sector.* Sustainability, 2013. **5**(2): p. 695-710.
9. Smith, A., Seyfang, G.,. *Introducing Grassroots Innovations for Sustainable Development*. 2010 [cited 2017 September 20]; Available from: https://grassrootsinnovations.files.wordpress.com/2012/03/gi-1-intro-briefing-small.pdf.
10. Church, C. and J. Elster, *The Quiet Revolution.* Shell Better Britain, Birmingham, 2002.

11. Devine-Wright, P., *Citizenship, responsibility and the governance of sustainable energy systems.* Framing the Present, Shaping the Future: Contemporary Governance of Sustainable Technologies Earthscan, London, 2006.
12. Fine, B., *Leopold E.(1993), The World of Consumption*. routledge, london.
13. Lakitan, B. *Role of Government in Energizing Grassroots Innovations*. in *Proceeding of International Seminar Enhancing Grassroots Innovation Competitiveness for Poverty Alleviation (EGICPA). Indonesia: Yogyakarta, October*. 2012.
14. Fressoli, M., et al., *When grassroots innovation movements encounter mainstream institutions: implications for models of inclusive innovation.* Innovation and Development, 2014. **4**(2): p. 277-292.
15. Foster, C. and R. Heeks, *Conceptualising inclusive innovation: Modifying systems of innovation frameworks to understand diffusion of new technology to low-income consumers.* The European Journal of Development Research, 2013. **25**(3): p. 333-355.
16. Organisation for Economic Co-operation and Development. *Innovation for Development. Discussion Report*. [cited 2017 September 20].
17. Wang, F., Wang, R., Sun, F., Li, Z., Liu, D., Zhao, Y., Liu, F., Guo, R., et al.,, *Overview of Inclusive Innovation Policies in the People's Republic of China*. 2016, Chinese Academy of Science and Technology for Development, Beijing.: Chinese Academy of Science and Technology for Development, Beijing.
18. Schot, J. and F.W. Geels, *Strategic niche management and sustainable innovation journeys: theory, findings, research agenda, and policy.* Technology analysis & strategic management, 2008. **20**(5): p. 537-554.

19. Smith, A. and R. Raven, *What is protective space? Reconsidering niches in transitions to sustainability.* Research policy, 2012. **41**(6): p. 1025-1036.

20. Wakeman, T. *East Anglia Food Link: an NGO working on sustainable food*. in *Grassroots Innovations for Sustainable Development conference, UCL London, 10th June*. 2005.

21. Boyle, D. *Sustainability and Social Assets: The potential of time banks and coproduction*. in *Grassroots Innovations for Sustainable Development conference, UCL London, 10th June*. 2005.

22. Schumpeter, J.A., *Schumpeter, J.A., 1934. The Theory of Economic Development: An Inquiry into Profits, Capital, Credit, Interest, and the Business Cycle*. 1934, Cambridge, MA: Harvard University Press.

23. Amabile, T.M., *A model of creativity and innovation in organizations.* Research in organizational behavior, 1988. **10**(1): p. 123-167.

24. Sternberg, R.J., *The nature of creativity.* Creativity research journal, 2006. **18**(1): p. 87-98.

25. Hunter, S.T., et al., *Applying multiple knowledge structures in creative thought: Effects on idea generation and problem-solving.* Creativity Research Journal, 2008. **20**(2): p. 137-154.

26. Sternberg, R.J., J.E. Pretz, and J.C. Kaufman, *Types of Innovations*, in *The International Handbook on Innovation*, L.V. Shavinina, Editor. 2003, Pergamon: Oxford. p. 158-169.

27. Shalley, C.E. and L.L. Gilson, *What leaders need to know: A review of social and contextual factors that can foster or hinder creativity.* The leadership quarterly, 2004. **15**(1): p. 33-53.

28. Amabile, T., *Creativity in context (Boulder, CO, Westview Press).* 1996.

29. Rogers, E.M., *Diffusion of Innovations the Free Press of Glencoe.* NY, 1962. **32**: p. 891-937.

30. Gerrard, P. and J. Barton Cunningham, *The diffusion of internet banking among Singapore consumers.* International journal of bank marketing, 2003. **21**(1): p. 16-28.

31. Rogers, E., *Diffusion of Innovations (4th Eds.) ACM The Free Press (Sept. 2001).* New York, 1995: p. 15-23.

32. Porter, M.E., *Competitive Strategy Free Press New York.* PorterCompetitive Strategy1980, 1980.

33. Giddens, A., *The constitution of society: Outline of the theory of structuration*. Vol. 349. 1986: Univ of California Press.

34. Feola, G. and A. Butt, *The diffusion of grassroots innovations for sustainability in Italy and G reat B ritain: An exploratory spatial data analysis.* The Geographical Journal, 2017. **183**(1): p. 16-33.

35. Jalas, M., et al., *Everyday experimentation in energy transition: A practice-theoretical view*. Journal of cleaner production, 2017. **169**: p. 77-84.

36. Rajan, P., *Organizing grassroots innovations: Examining knowledge creation and sharing practices for technological innovation at the grassroots*. 2012, Purdue University.

37. Dana, L.P. and T.E. Dana, *Expanding the scope of methodologies used in entrepreneurship research.* International Journal of Entrepreneurship & Small Business, 2005. **2**(1): p. 79-88.

38. Seyfang, G., *Working for the Fenland dollar: an evaluation of local exchange trading schemes as an informal employment strategy to tackle social exclusion.* work, employment and society, 2001. **15**(3): p. 581-593.

39. Seyfang, G., *Ecological citizenship and sustainable consumption: Examining local organic food networks.* Journal of rural studies, 2006. **22**(4): p. 383-395.

40. Seyfang, G., *Harnessing the potential of the social economy? Time banks and UK public policy.* International journal of sociology and social policy, 2006. **26**(9/10): p. 430-443.

41. Seyfang, G., *Cultivating carrots and community: local organic food and sustainable consumption.* Environmental values, 2007: p. 105-123.

42. Yin, R.K., *Case study research: design and methods 5th ed.* Thousand Oaks, 2014.

43. Dana, L.P., *An analysis of strategic intervention policy in Namibia.* Journal of Small Business Management, 1993. **31**(3): p. 90.

44. Mitlin, D. and A. Muller, *Windhoek, Namibia: towards progressive urban land policies in Southern Africa.* International Development Planning Review, 2004. **26**(2): p. 167-186.

45. *Namibia Unemployment Rate*. [cited 2017 September 20]; Available from: https://tradingeconomics.com/namibia/unemployment-rate

46. Steytler, D., *The Namibia Labour Force Survey 2012 Report.* Windhoek Namibia: Namibia Statistics Agency (NSA), 2013.

47. Laschinger, H.K., Purdy, N., Almost, J.,, *The Impact of Leader-Member Exchange Quality, Empowerment, and Core Self-evaluation on Nurse Manager's Job Satisfaction.* The Journal of Nursing Administration, 2007. **35**(5): p. 221-229.

48. Muller, A., Mbanga, E., , *Participatory enumerations at the national level in Namibia: the Community Land Information Programme (CLIP).* Environment and Urbanization, 2012. **24**(1): p. 67-75.

49. Allard-Poesi, F., *Coding the data. In: Giordano, Y. (Ed.), Conduct a research project in a qualitative perspective.* EMS, 2003: p. 245-290.

50. Lincoln, Y.S., Guba E.G., *Naturalistic Inquiry*. 1985, Beverly Hills, CA.

51. Barbieri, J., F. Riva, and E. Colombo, *Cooking in refugee camps and informal settlements: a review of available technologies and impacts on the socio-economic and environmental perspective.* Sustainable Energy Technologies and Assessments, 2017. **22**: p. 194-207.

52. Palmer, C. and J. MacGregor, *Fuelwood scarcity, energy substitution, and rural livelihoods in Namibia.* Environment and Development Economics, 2009. **14**(6): p. 693-715.

53. Schenck, W., *Men at the Side of the Road – Paper Brick Project.* 2012: Nuevas Ideas Consulting.

54. Domingue, E.C., et al., *SUSTAINABLE PAPER INSULATION FOR KAMBASHUS IN INFORMAL SETTLEMENTS OF NAMIBIA.* 2012.

55. Woodyard, A.H., et al., *THE PAPER BLOCK PROJECT: IMPROVING THE DESIGN AND IDENTIFYING MARKET OPPORTUNITIES FOR A SUSTAINABLE COOKING FUEL IN KATUTURA, NAMIBIA.* 2014.

56. Dovey, K. and R. King, *Forms of informality: Morphology and visibility of informal settlements.* Built Environment, 2011. **37**(1): p. 11-29.

Specific Aim 3: Chapter 8 Utilizing the Lean Startup Methodology and Business Model Canvas as Tools to Chart a Path to Market for AMP Based Technologies

8.1 A path to market for AMP based technologies

Commercialization of AMPs has been of great interest due to the growing problem of antimicrobial resistance [1-10]. Commercialization of novel therapeutics is a challenging and expensive process [10-13]. Currently the net present value (NPV) to develop new antibiotics drugs is negative, with an average cost to reach market of $1.4 billion [14, 15]. This presents a unique challenge due to the fact that the large pharmaceutical companies, that have the resources to develop these drugs, are not incentivized to do so because of the NPV [14-16]. Due to this, the number of large pharmaceuticals developing novel antimicrobials is small placing the burden of development onto academia and startups [15, 16]. Costs for pre-human trials account for approximately 30% of the drug development process, academic institutions and start-ups can play a role in reducing these costs [15]. Many incentive programs for the development of antimicrobials are available for early stage start-ups and small companies [11]. When it comes to academic spin outs (start-ups), students are more than twice as likely to start a company compared to tenured faculty [17]. Thus when talking about academic involvement in commercializing novel antimicrobial technologies, there should be a focus on students [17]. We previously demonstrated the benefits of entrepreneurial training and education [18].

One of the major components of a start-up company is the business plan, seen by many as a key element for obtaining an investment [19-21]. There is a debate to the necessity of the traditional business plan model, and even with entrepreneurship training many Ph.D. students will find it difficult to put together a traditional business plan [19, 21, 22]. However, all entrepreneurs need to be able to answer key questions whose answers are found within the business plan, such as defining the customer need [20, 22]. A new alternative to the traditional business plan is *The Business Model Canvas* (BMC), Figure 1, developed by Alexander Osterwalder [21]. The BMC

contains many of the traditional business plan's core elements in a format that is easy to visualize, intuitive to use, and is based on the Lean Startup methodology [21-24]. The Lean Startup methodology is rooted in the lean manufacturing methodology developed at Toyota, with progress measured through validated learning [23, 25]. Defined in other way, the Lean Startup methodology is the intersection of customer development and agile development [25, 26]. The BMC is more adaptable, allowing for pivots to be made quickly and easily, which is required of an early stage start up trying to find a product market fit [20, 21, 27]. The right side of the BMC is focused on the business activities surrounding the customer, while the left side focuses on the internal activities of the business [20-22]. More specifically, the BMC has the following sections [21]:

Right Side of the Canvas

1) Customer Segments, defines the people or groups the business aims to reach and serve
2) Value Propositions, defines the differentiating factors that bring value to the customer
3) Channels, defines how the company communicates and reaches their Customer Segments
4) Customer Relationships, defines how the Company and Customer interact with each other
5) Revenue Streams, defines how the company generates cash from each Customer Segment

Left Side of the Canvas

6) Key Resources, defines the most important assets needed to make the business model work
7) Key Activities, defines the most important activities needed to make the business model work
8) Key Partnerships, describes the network of suppliers and partners that make the business model work
9) Cost Structure, describes the costs incurred to operate the business model

Assumptions (hypotheses) are made and placed in the business model canvas, for example the customer will find X feature desirable because of Y [20-22]. These assumptions are tested by talking to experts and customers [13, 20, 22, 24]. If an assumption is confirmed it remains on the canvas, if it is rejected a new hypothesis is added based on the new information and then tested [20-22]. This iterative process is then continued until all the hypothesis are confirmed and there are no unanswered questions, at this point a scalable business model has been developed [20-22]. The I-Corps program has had thousands of teams use these tools to great success which addresses the concern of training students to put together the BMC [24].

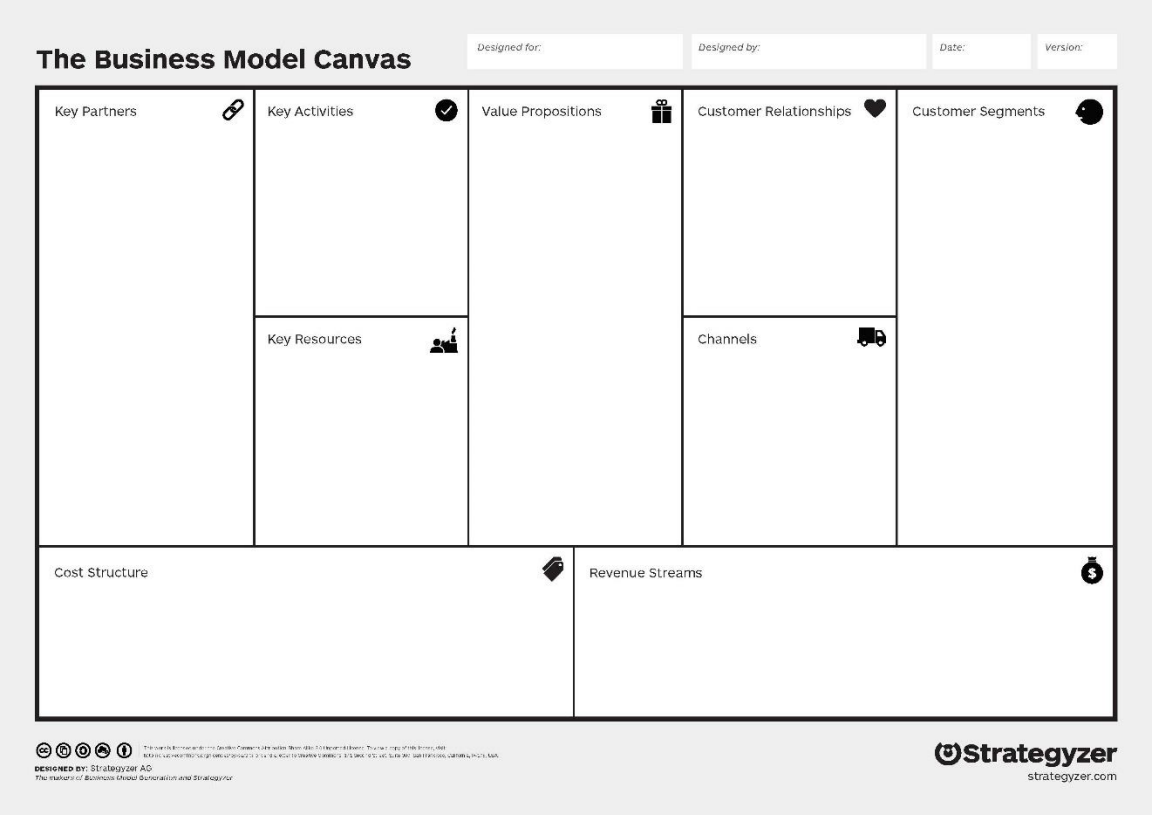


Figure 1*:* The Business Model Canvas as developed by Alexander Osterwalder. It allows for a quick visualization of all the key elements of a business plan. This allows for rapid iteration as well as visual representation of the gaps that need to be filled.

The BMC allows for the formation and testing of hypotheses and quick iterations that are important to developing a scalable, sustainable business model [20, 21, 28]. The Innovation

Corps (I-Corps) program at the National Science Foundation (NSF) for startups and commercial ideas relies heavily on the BMC and lean startup methodology [24]. While only 14% of researchers who apply for a small business innovation research (SBIR) grant receive funding, 60% of applicants who went through the I-Corps program receive SBIR funding [29]. Even though the BMC is a relatively new model, it has proven to be effective and therefore a business model canvas was developed for this project with results that can be extended to other AMP-based products. In order to test hypotheses in each of the BMC categories and iterate on the BMC, there were over 250 customer interviews performed, including 100 during the NSF I-Corps program [27, 30].

When looking at the challenge of translating AMPs from the benchtop to the clinical bedside, it is important to thoroughly understand why they have not successfully made it to the clinic thus far in order to avoid pitfalls [3, 7, 9, 31, 32]. A few challenges associated with AMPs are their cytotoxicity (translated as a low therapeutic ratio) [3, 6-8, 33]; their stability in vivo [3, 6-8, 31, 34]; due to initial electrostatic interaction, many AMPs are ineffective at physiologically relevant salt conditions [31, 35-39]; and their cost, which is exacerbated by the low half-life in vivo [9, 40]. These technical challenges were covered in the background section more thoroughly, and in Costa *et al.* [3]. To circumvent most of these issues, we decided to covalently binding the peptide to a surface when combined with the salt tolerant cysteine modified Chrysophsin-1, C-CHY1 [31, 41-45].

When choosing an initial target market three major factors were taken into consideration:

1) There was an unmet clinical need
2) The market size was $1 billion dollars or more
3) Additional cost of the C-CHY1 coating was economical

With the three above mentioned criteria in mind, we decided that the orthopedic knee and hip markets were ideal initial targets for the C-CHY1 coating to prevent infection. Over 600,000 joint prosthesis are implanted each year and the number keeps growing significantly—with an infection rate of 1-2% of implanted devices [46-49]. This cost the US an excess of $500 million a year to treat these infections, at an average cost of $50,000 to $75,000 per case [47, 48]. The gold standard of treatment is a revision surgery which involves removal of the implant and debridement, causing significant pain and discomfort, and the use of traditional antibiotics which promotes resistance [47, 50]. Even worse, those who experience a prosthetic joint infection have a mortality rate five times higher than those without infection [47, 51]. Based on the literature, this seemed like an ideal market to target. In 2014 we applied the NSF I-Corps Program and were accepted into the Washington D.C. node. Our initial BMC with our hypotheses is displayed in Figure 2A. As can be seen by the first version of the canvas, our hypotheses were broad and unrefined. As we completed more interviews, our questions and hypotheses became more refined. During the seven week I-Corps program we talked to 100 potential customers including orthopedic surgeons, device manufacturers, nurses, patients, and many other people in the ecosystem [20, 22].

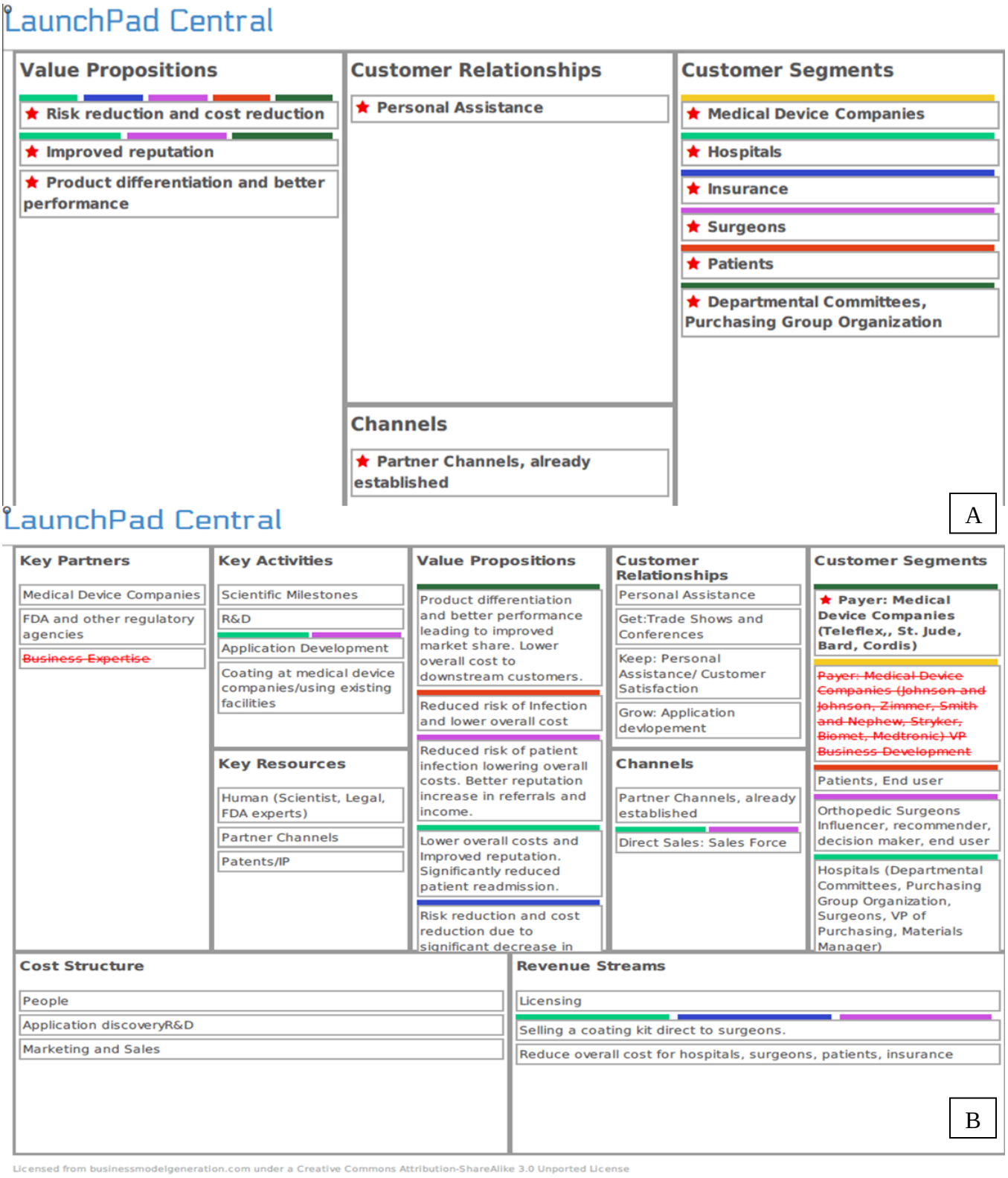


Figure 2: A) Business model canvas at the start of the NSF I-Corps program, March 2014. Our initial hypotheses were very general. Value propositions were not specific. No product market fit.

B) Business model canvas at the end of the NSF I-Corps program, May 2014. More refined value proposition, specific. Product market fit refined.

Approximately halfway through the program we found through our interviews, that our hypothesis that orthopedic knees and hips would be a good market for AMPs was true. However, it became clearer through our interviews that urinary catheters, in particular Foley urinary catheters, would be a better fit for our AMP based coating. This led to a significant change in our BMC, Figure 2B. The reasons that the various experts, customers, and users gave for why Foley catheters were a better fit than orthopedic knees and hips were as follows:

1) Higher incidence of infection >5% versus 1-2%
2) Number one cause of hospital acquired infections
3) Never event (un-reimbursable by Medicare and Medicaid)
4) 510K approval pathway likely versus PMA with orthopedics

Due to the interviews we conducted during the NSF I-Corps program we pivoted from our initial idea as a coating for orthopedic implants to one for Foley urinary catheters. In lean start up terms, we found our product market fit [20, 22]. We continued to refine our hypothesis and understanding of the competitive landscape. We participated in the VentureWell E-Team program that also used the BMC, followed the lean start up methodology, and required customer interviews [20-22, 52]. Changes throughout the course of the E-teams program can be seen below in Figure 3. In this case we can see further refinement of the business model itself even though the target market remained the same. We further refined our customer segment and our path to market. By talking to regulatory consultants we were able to confirm a 510K path to market.

Key Partners	Key Activities	Value Proposition(s)	Customer Relationships	Customer Segments
Lawyer/Law Firm	Research and Development	Antimicrobial Coating	Direct Contact/marketing	Catheter Manufactueres (Bard, Boston Scientific, Teleflex)
Government Agencies (Funding, Regulatory)	IP & Patent Portfolio	Differentiation over Competition	Scientific Validation	~~End Users (Surgeons, nurses, patients)~~
Regulatory Consultants (InSymbiosis)	Trade Shows, Conferences	Non-Leaching	"Any" Connection Mangemnt	~~Influencers (Surgeons, nurses, medicare)~~
	Talking to end users	Does not promote resistance	R&D, M&A, Mfg.	Payers Hospitals (PGOs, purchasing departments) (Discusion should center around VP)
	Reimbursement Estimates	Broad-Spectrum	Leagal	Influencers (Surgeons, medicare)
		Favorable Regulation Classification		End Users (Surgeons, patients)
	Sterilization (?)	Reduced LOS, 80% reduction in CAUTIs		
		Reduction of never events		
	Key Resources	Non-Toxic	**Sales Channels**	
	Human (Scientists,Legal)		Technology Transfer Office	
			Distributors	
	Sterilization (?)		Distributor - McKesson	

Cost Model	Revenue Model
Research and Development	Licensing (one time payments and recurring royalties)
Legal (patents, representation)	
Scientists/human resources	
Animal Studies	*Pricing Model*
Clinical Trials	
Medical Device Taxes	
Regulatory Consultant	

A

Suppliers and Partners	Key Activities	Value Proposition(s)	Customer Relationships	Customer Segments
		link value props to aligned customer segments, sales channels, revenue models etc w/matching font color(s)		
Lawyer/Law Firm	Research and Development	Antimicrobial Coating	Direct Contact/marketing	Catheter Manufactueres (Bard, Boston Scientific, Teleflex)
Government Agencies (Funding, Regulatory)	IP & Patent Portfolio	Differentiation over Competition	Scientific Validation	Payers Hospitals (PGOs, purchasing departments) (Discusion should center around VP)
Regulatory Consultants (InSymbiosis)	Trade Shows, Conferences		"Any" Connection Mangemnt	Influencers (Surgeons, medicare)
	Talking to end users	Does not promote resistance	R&D, M&A, Mfg.	End Users (Surgeons, patients)
Contract Research Organizations (CROs)	Reimbursement Estimates	Broad-Spectrum	Legal	
		Favorable Regulation Classification		Dialysis (DaVita, Fresenius [70% US market])
	Sterilization	Reduced LOS, 80% reduction in CAUTIs		
		Reduction of never events		
	Key Resources	Non-Toxic	**Sales Channels**	
	Human (Scientists, Legal)		Technology Transfer Office	
		Antifouling ?	Distribuors	
		Non-Leaching		
			Distributor - McKesson	
	Sterilization			

Cost Model	Revenue Model
Research and Development	Licensing (one time payments and recurring royalties)
Leagal (patents, representation)	
Scientists/human resources	
Animial Studies	*Pricing Model*
Clinical Trials	
Medical Device Taxes	
Regulatory Consultant	

B

Figure 3: A) Business model canvas at the start of E-Team Stage 1, July 2015, B) Business model canvas at the end of E-Team Stage 2 April 2016. Both demonstrating the refinement of the business model canvas overtime.

The BMC allowed us to rapidly test hypotheses, change or remove hypotheses that were proven untrue, and have a good visual way to look at our plan. From this we developed a more refined business plan, Supplemental Section. One example was the hypothesis that surgeons were decision makers in the choice of what Foley catheters to use, Figure 1. What we found was that this was not true, the hospitals Purchasing Departments (or Materials Acquisition) are the major decision makers and that surgeons may influence the process but do not have the final say, Figure 2. We found that this is partially due to the fact that many "commodity" supplies are bundled and thus sometime there is also limited choice, Figure 2. Iterating on our hypotheses led to a plan that is scalable for our specific coating, but the lean start-up methodology and BMC technique we used can be applied to other AMP based products.

The entrepreneurial education that we received through formal coursework, training programs (I-Corps, VentureWell), and through our mentors proved invaluable to this venture. However, we must be cautious about generalization due to this being a single case study [18]. Engineering schools and universities are extremely successful at producing highly skilled technically proficient graduates [27]. With emerging global threats such as antimicrobial resistance, we present a case study that demonstrates that in this case higher education can serve both the needs of the student and societal needs [10, 18, 50, 53]. We call for more research to determine if this theory holds up under other circumstances [18]. For the student, this includes both technical as well as entrepreneurial training and education which better prepares them to tackle real world problems in the workforce [18, 27]. Society also benefits as scientists and engineers are uniquely poised to bridge the gap "between human knowledge and human need" [27]. Our research was improved by the entrepreneurial study, from merely having a "broader impact" to understanding how we can have the broadest impact by figuring out where our technology can have the biggest influence. We

successfully used the BMC and Lean Startup methodology in order to improve our work [20-22, 24]. The Lean Startup methodology and BMC are easily introduced to and used by scientist and engineers because they are familiar with the ideas of proposing, testing, and iterating hypotheses [20-22, 24].

A new model for training engineering graduate students, specifically in the healthcare sector, was developed at Johns Hopkins University and is called "Spiral Innovation" [27]. The Center for Bioengineering Innovation & Design (CBID), borrows concepts from the Lean Startup methodology and has been met with great success [22, 27]. In the 4 years of operation, 61 total students, 5 startups were launched ($2.5 in funding), and 25 patents filled [27]. While our particular case is just one example —and a positive symptom of the NSF IGERT program at Worcester Polytechnic Institute—many other colleges and universities are recognizing the synergy and benefits between engineering and entrepreneurship [18, 54]. Additionally, the number of student entrepreneurs are increasing and many universities are starting to recognize and support that by developing programs that allow for students to both remain and student and be an entrepreneur [54, 55]. We have shown that the Lean Startup methodology, the BMC, and entrepreneurial education benefits graduate student education [18, 27, 53, 54]. Approximately 90% of start-ups fail, not all students that receive entrepreneurial education form a start-up, but the benefits of an entrepreneurial mindset are vast [18, 27, 53, 54]. This case study helps highlight some of those befits including, comfortability in developing a business model, understanding customer needs, and allows for students to use their engineering knowledge creatively.

8.2 References

1. O'Neill, J., *Infection prevention, control and surveillance: limiting the development and spread of drug resistance. The review on antimicrobial resistance*. 2016.
2. O'Neill, J., *The review on antimicrobial resistance. Tackling a global health crisis: initial steps*. 2015, AMR Review, London.
3. Costa, F., et al., *Clinical Application of AMPs*, in *Antimicrobial Peptides: Basics for Clinical Application*, K. Matsuzaki, Editor. 2019, Springer Singapore: Singapore. p. 281-298.
4. Trusts, T.P.C. *Antibiotics Currently in Global Clinical Development*. Antibiotic Resistance Project 2018 [cited 2018 Jun 11]; Available from: http://www.pewtrusts.org/-/media/assets/2018/03/antibiotics_clinical_dev_table_february2018.pdf?la=en&hash=D1E1FEAC1B30A1A7721FE6A5428C73EF122B2B8F.
5. Butler, M.S., M.A. Blaskovich, and M.A. Cooper, *Antibiotics in the clinical pipeline at the end of 2015.* The Journal of antibiotics, 2017. **70**(1): p. 3-24.
6. Wimley, W.C. and K. Hristova, *Antimicrobial Peptides: Successes, Challenges and Unanswered Questions.* The Journal of Membrane Biology, 2011. **239**(1): p. 27-34.
7. Jenssen, H., P. Hamill, and R.E.J.C.m.r. Hancock, *Peptide antimicrobial agents.* 2006. **19**(3): p. 491-511.
8. Eckert, R., *Road to clinical efficacy: challenges and novel strategies for antimicrobial peptide development.* Future microbiology, 2011. **6**(6): p. 635-651.
9. Zasloff, M., *Antimicrobial Peptides: Do They Have a Future as Therapeutics?* 2016. p. 147-154.

10. O'Neill, J., *Antimicrobial resistance: tackling a crisis for the health and wealth of nations.* Rev. Antimicrob. Resist, 2014. **20**: p. 1-16.

11. Simpkin, V.L., et al., *Incentivising innovation in antibiotic drug discovery and development: progress, challenges and next steps.* The Journal of antibiotics, 2017. **70**(12): p. 1087-1096.

12. Baycan, T. and R.R. Stough, *Bridging knowledge to commercialization: the good, the bad, and the challenging.* The Annals of Regional Science, 2013. **50**(2): p. 367-405.

13. Huang-Saad, A., J. Fay, and L. Sheridan, *Closing the divide: accelerating technology commercialization by catalyzing the university entrepreneurial ecosystem with I-Corps™.* The Journal of Technology Transfer, 2017. **42**(6): p. 1466-1486.

14. Kimberly Sciarretta, A.O., Adrianus Van-Hengel, John-Arne Rottingen, Joseph Larsen, *Economic Incentives for Antimicrobial Therapy Development: Summary from the Transatlantic Task Force on Antimicrobial Resistance*. 2016: Centers for Disease Control.

15. DiMasi, J.A., H.G. Grabowski, and R.W. Hansen, *Innovation in the pharmaceutical industry: New estimates of R&D costs.* Journal of Health Economics, 2016. **47**: p. 20-33.

16. Luepke, K.H., et al., *Past, present, and future of antibacterial economics: increasing bacterial resistance, limited antibiotic pipeline, and societal implications.* Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 2017. **37**(1): p. 71-84.

17. Åstebro, T., N. Bazzazian, and S. Braguinsky, *Startups by recent university graduates and their faculty: Implications for university entrepreneurship policy.* Research Policy, 2012. **41**(4): p. 663-677.

18. Alexander, T.E., Lozeau, Linsday D., Camesano, Terri A., Hoy, Frank, *Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program.* Portland International Center for Management of Engineering and Technology (PICMET), 2019.

19. Ashamalla, M.H., J.N. Orife, and I. Abel, *Business Plans: Are They Relevant to Venture Capitalists?* Journal of Small Business & Entrepreneurship, 2008. **21**(4): p. 381-391.

20. Blank, S. and B. Dorf, *The startup owner's manual: The step-by-step guide for building a great company*. 2012: BookBaby.

21. Osterwalder, A. and Y. Pigneur, *Business model generation: a handbook for visionaries, game changers, and challengers*. 2010: John Wiley & Sons.

22. Blank, S., *Why the lean start-up changes everything.* Harvard business review, 2013. **91**(5): p. 63-72.

23. Ries, E., *The lean startup methodology.* The Lean Startup, 2012.

24. Blank, S. and J. Euchner, *The Genesis and Future of Lean Startup: An Interview with Steve Blank.* Research-Technology Management, 2018. **61**(5): p. 15-21.

25. Ries, E., *The lean startup: How today's entrepreneurs use continuous innovation to create radically successful businesses*. 2011: Crown Books.

26. Blank, S., *What's A Startup? First Principles.* 2010: https://steveblank.com/.

27. Yazdi, Y. and S. Acharya, *A New Model for Graduate Education and Innovation in Medical Technology.* Annals of Biomedical Engineering, 2013. **41**(9): p. 1822-1833.

28. York, J., *Putting Lean Startup into Perspective: A Novel Approach for Discovering and Developing a Successful Business Model.* Arch Bus Adm Manag: ABAM-104. DOI, 2018. **10**.

29. Millette, E., *Steve Blank.* 2014.

30. *National Science Foundation - Where Discoveries Begin.* [cited 2019; Available from: https://www.nsf.gov/news/special_reports/i-corps/.

31. Lozeau, L.D., T.E. Alexander, and T.A. Camesano, *Proposed mechanisms of tethered antimicrobial peptide chrysophsin-1 as a function of tether length using QCM-D.* The Journal of Physical Chemistry B, 2015. **119**(41): p. 13142-13151.

32. Reddy, K., R. Yedery, and C. Aranha, *Antimicrobial peptides: premises and promises.* International journal of antimicrobial agents, 2004. **24**(6): p. 536-547.

33. Matsuzaki, K., *Control of cell selectivity of antimicrobial peptides.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2009. **1788**(8): p. 1687-1692.

34. Cloutier, M., D. Mantovani, and F. Rosei, *Antibacterial Coatings: Challenges, Perspectives, and Opportunities.* Trends in Biotechnology, 2015. **33**(11): p. 637-652.

35. Li, X., et al., *Antimicrobial functionalization of silicone surfaces with engineered short peptides having broad spectrum antimicrobial and salt-resistant properties.* Acta biomaterialia, 2014. **10**(1): p. 258-266.

36. Sperstad, S.V., et al., *Antimicrobial peptides from marine invertebrates: Challenges and perspectives in marine antimicrobial peptide discovery.* Biotechnology Advances, 2011. **29**(5): p. 519-530.

37. Chu, H.-L., et al., *Boosting Salt Resistance of Short Antimicrobial Peptides.* Antimicrobial Agents and Chemotherapy, 2013. **57**(8): p. 4050.

38. Tam, J.P., Y.-A. Lu, and J.-L. Yang, *Correlations of cationic charges with salt sensitivity and microbial specificity of cystine-stabilized β-strand antimicrobial peptides.* Journal of Biological Chemistry, 2002. **277**(52): p. 50450-50456.

39. Han, H.M., R. Gopal, and Y. Park, *Design and membrane-disruption mechanism of charge-enriched AMPs exhibiting cell selectivity, high-salt resistance, and anti-biofilm properties.* Amino Acids, 2016. **48**(2): p. 505-522.

40. Zasloff, M., *Antimicrobial peptides of multicellular organisms.* nature, 2002. **415**(6870): p. 389.

41. Yu, K., et al., *Anti-adhesive antimicrobial peptide coating prevents catheter associated infection in a mouse urinary infection model.* Biomaterials, 2017. **116**: p. 69-81.

42. Lozeau, L.D., et al., *Collagen tethering of synthetic human antimicrobial peptides cathelicidin LL37 and its effects on antimicrobial activity and cytotoxicity.* 2017. **52**: p. 9-20.

43. Li, Y., et al., *Effects of peptide immobilization sites on the structure and activity of surface-tethered antimicrobial peptides.* The Journal of Physical Chemistry C, 2015. **119**(13): p. 7146-7155.

44. Hilpert, K., et al., *Screening and characterization of surface-tethered cationic peptides for antimicrobial activity.* Chemistry & biology, 2009. **16**(1): p. 58-69.

45. Onaizi, S.A. and S.S. Leong, *Tethering antimicrobial peptides: current status and potential challenges.* Biotechnology advances, 2011. **29**(1): p. 67-74.

46. Darouiche, R.O., *Treatment of infections associated with surgical implants.* New England Journal of Medicine, 2004. **350**(14): p. 1422-1429.

47. Tobin, E.J., *Recent coating developments for combination devices in orthopedic and dental applications: A literature review.* Advanced Drug Delivery Reviews, 2017. **112**: p. 88-100.

48. Kazemzadeh-Narbat, M., et al., *Antimicrobial peptides on calcium phosphate-coated titanium for the prevention of implant-associated infections.* Biomaterials, 2010. **31**(36): p. 9519-9526.

49. Arciola, C.R., et al., *Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials.* Biomaterials, 2012. **33**(26): p. 5967-5982.

50. Centers for Disease Control and Prevention, *Antibiotic resistance threats in the United States, 2013. Atlanta: CDC; 2013*. 2014.

51. Zmistowski, B., et al., *Periprosthetic joint infection increases the risk of one-year mortality.* JBJS, 2013. **95**(24): p. 2177-2184.

52. VentureWell. *e-team grant program*. Available from: https://venturewell.org/e-team-grant-program/.

53. Yazdi, Y., *Developing innovative clinicians and biomedical engineers: a case study.* American journal of preventive medicine, 2013. **44**(1): p. S48-S50.

54. Manbachi, A., et al., *Starting a Medical Technology Venture as a Young Academic Innovator or Student Entrepreneur.* Annals of Biomedical Engineering, 2018. **46**(1): p. 1-13.

55. Yetisen, A.K., et al., *Entrepreneurship.* Lab on a Chip, 2015. **15**(18): p. 3638-3660.

Business Plan

BUSINESS SUMMARY

AMProtection, LLC

Contents

I. Company Overview and Mission Statement (pp. 2)
II. Management Team and Advisors (pp. 3-4)
III. Value Proposition of Novel Technology (pp. 5)
IV. Market Opportunity and Addressable Patient Population (pp. 6)
V. Pricing and Reimbursement Strategies (pp. 7)
VI. Commercialization Approach (pp. 8-9)
VII. Fundraising Goals, Milestones and Value Inflection Points (pp. 10)
VIII. Financial Projections (pp. 11)
IX. Compelling Preliminary Data (pp. 12-13)
X. An Overview of the Most Current Scientific Aims (pp. 14-16)
XI. Other Media (pp. 16)

Company Website

http://amprotection.us

Contacts

Lindsay D. Lozeau (Co-Founder)

Todd E. Alexander (Co-Founder)

I. Company Overview and Mission Statement

AMProtection, LLC is a university startup currently operating in laboratory space at Worcester Polytechnic Institute's (WPI) Gateway Park in central Massachusetts. The founders are two WPI graduate students and their faculty advisor, mentored by many local scientific and business experts. AMProtection is a technology-based company in the biomanufacture of antimicrobial peptide (AMP) coatings for a wide variety of medical devices and a wide variety of antimicrobial uses.

AMProtection, LLC was founded in August 2016 with a vision to become the premier biomedical technology company offering platform coating technologies to prevent antimicrobial resistance. Our aim is to *"develop tomorrow's antimicrobials, today."*

AMProtection, LLC is predominantly women-owned, and the founders are dedicated to promoting innovation and entrepreneurship in STEM education for young students, especially women. From our technology's NSF lineage and company's WPI origin, we have developed important company values. These values are (1) promoting engineering education, (2) teaching the entrepreneurial mindset, (3) mentoring other entrepreneurs, (4) emphasizing the importance of the customer interview process and (5) raising awareness for the antimicrobial resistance crisis.

Our five-year vision involves the extension of our coating technology into multiple channels and antimicrobial indications by expanding our patent portfolio to other AMP coating technologies. We plan to first commercialize a coating for preventing catheter-associated urinary tract infection (CAUTI) on urinary catheters and then to continue developing platform for the next highest need catheter segments (e.g. central venous catheters, $300 million U.S. market and peripherally-inserted central $850 million global market by 2019). Orthopedic knee and hip implant-associated infections are also devastating (U.S. market, $5 billion) and are another potential market for the AMProtection technology.

II. Management Team and Advisors

Lindsay D. Lozeau received her Ph.D. in Chemical Engineering and a business certificate in entrepreneurship from Worcester Polytechnic Institute (WPI), where she was a NSF IGERT and WPI Hitchcock fellow, published in peer-reviewed journals, presented at national and international conferences, founded a graduate student organization, and won several elevator pitch competitions. She performed several customer and value chain discovery for AMProtection under three VentureWell E-team programs. She has mentored several hundred engineering students and several student projects at WPI. In addition to co-developing the IP for this tethered antimicrobial peptide (AMP) system, she performed successful work on a Phase I NSF STTR involving another patented AMP. She spent three years at Kimball Physics, Inc. in micromanufacturing, quality testing, R&D of cathodes and electron guns and implemented training of incoming physicists. At TEI Biosciences (now Integra) she worked in R&D for wound healing and reconstructive scaffolds. At AMProtection she is responsible for overseeing technical milestones and full-time R&D.

Todd E. Alexander has a B.S. in Chemical Engineering from WPI and will earn his Ph.D. in Innovation & Entrepreneurship in Biomolecular Engineering in May 2018. He also held NSF IGERT and WPI Hitchcock fellowships, and was entrepreneurial lead on the NSF I-Corps team, which significantly advanced the fundamental and commercial research behind the AMProtection technology. He was involved with a Kern Family Foundation KEEN grant initiative to catalyze innovation and entrepreneurship in engineering education at WPI. Todd worked for

Teleflex, developing anti-adhesive coatings for endotracheal tubes, and Karl Storz in fiber optics. At AMProtection, Todd is responsible of business development, market and customer validation.

Dr. Terri Camesano is Dean of Graduate Studies and a Chemical Engineering professor with over 10 years' experience working with basic and translational AMP technologies. She has been PI of several related projects, including NSF IGERT and NSF I-Corps and was advisor on the I-Corps team. She helped develop these ideas into a business model, and her laboratory has been involved with the development of unique, patented technologies based on surface-tethered AMPs for many years.

Advisors

- **Frank Hoy, Ph.D.**, Paul R. Beswick Professor of Innovation and Entrepreneurship and director of the Collaborative for Entrepreneurship & Innovation at WPI. He was co-PI on IGERT and the I-Corps team mentor, who has experience launching and growing several entrepreneurial ventures.
- **Dr. Douglas Waite, M.D.,** Chief Medical Officer (CMO) at Covidien Health, and has been System CMO of Day Kimbell Healthcare. He specializes in infectious disease pathology, has expertise in hospital purchasing systems, and with his passion for reducing healthcare-associated infection, advises the team on outcome-based experimental design.

- **Dr. Mitchell Sokoloff, M.D.,** founding urology department Chair at UMass Medical School in Worcester, MA, specializes in treatment of prostate, kidney and testis cancer. His 20 years of urology experience provides invaluable advice of clinical design, hospital purchasing, and procedures.
- **Yael Schwartz, Ph.D.,** President and CEO of Or-Genix Therapeutics, is a successful serial entrepreneur in the commercialization of biological, dermatology, and metabolic-derived products. She has seen several technologies through to commercialization and experienced successful exits. She is a well-known FDA and regulatory expert in the biological space and is WPI's Entrepreneur-in-Residence.
- **Mary McNamara, R.A.C.,** regulatory affairs partner at Alira Health and consultant has been involved in over 200 FDA 510k IND submissions. Her expertise is in submissions involving antimicrobial and wound care products. Mary works with the team to develop a strategic regulatory plan.
- **Jerold Shapiro, Ph.D.,** CEO of Fem-medical, LLC, is highly experienced in the incontinence products market. His expertise is in clinical design for urologic devices and designing FDA trials.

Other Advisors to the Team:

- **James A. Boiani, J.D.,** Regulatory Lawyer and member at Epstein, Becker & Green has experience in correspondence with FDA for devices and pharmaceuticals from pre-IND/IDE meetings through regulatory approval.
- **Mary Moccia, FNP-C** at Nashua Skin and Laser Surgery in Nashua, NH is an experienced clinician in the dermatology and laser surgery space for medical and cosmetic applications, has been advising the team in development of acne-related formulations.
- **Andrew D. Myers, J.D., M.B.A.,** shareholder at Davis, Malm & D'Agostine and member of the Boston Harbor Angels, is our business operations legal counsel.

- **Ray Knox, M.B.A**, Chief Engineering Officer at Lyndra, is a manufacturing, quality assurance, operations and R&D expert with experience taking products from prototypes to the market.
- **Jennifer Almy**, Principal Consultant CRC Quality Solutions, is a quality and compliance expert with significant experience in developing quality and compliance systems.
- **Jerome Schaufeld,** Professor of Practice in Business at WPI, has significant industry experience as a chief operations officer, hospital consultant, founder, investment firm director, and is author of "Commercializing Innovation, Turning Technology Breakthroughs into Products" (2015). He specializes in technology commercialization, operations, business model development and commercial validation.
- **Todd S. Keiller,** Director, Intellectual Property and Innovation, Worcester Polytechnic Institute, is an expert in licensing and marketing technologies. He currently helps AMProtection file and protect intellectual property.

III. Value Proposition of Novel Technology

The Need

With an economic burden of $1 billion annually in the U.S. ($1000 per incidence), catheter-associated urinary tract infections (CAUTIs) have considerable market pain points. In addition to reduced patient quality of life, CAUTIs increase hospital stays by 0.63 days and are classified as unreimbursed healthcare expenses called *never events* – hospital acquired conditions that never should have happened. Further, the rise of antibiotic resistance threatens efficacy traditional antibiotics. The unaddressed clinical and economic burdens of CAUTIs, as well as the grand challenge of addressing antibiotic resistance create tangible opportunities for AMProtection to introduce its platform antimicrobial technologies.

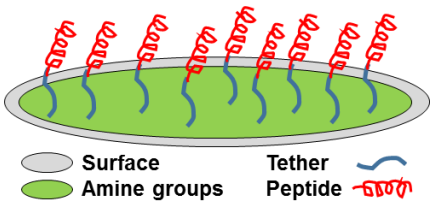


Figure 1: The AMProtection technology. The coating is made of AMPs covalently-bound to free amines on a surface via poly(ethylene glycol) "tethers".

The Novel Technology

The AMProtection innovation is a patent-pending, novel therapeutic agent – a naturally derived AMP that is covalently bound ("tethered") onto surfaces (**Fig. 1**). This AMP coating is biocompatible, is broad-spectrum, and kills bacteria directly, clearing infection through unique biophysical mechanisms. It is substantially differentiated from current catheter products that use the ineffective release of antibiotics or silver, because it is covalently bound ("tethered") uniformly onto catheter surfaces. Further, our research indicates our product will be a reimbursable, FDA Class II 510k medical device. Tethering of AMPs onto catheter surfaces is a non-traditional strategy of using AMPs; it allows maximized antimicrobial activity at the surface of the device where infection is most likely to occur.

The Value Proposition

Competition among catheter manufacturers to differentiate their antimicrobial urinary catheter products will escalate as antibiotic resistance worsens and CAUTI *never event* costs increase. The unaddressed clinical and economic burdens of CAUTIs, as well as the grand challenge to address antibiotic resistance, create tangible opportunities for AMProtection to introduce its platform antimicrobial technologies. Our patent-pending antimicrobial coating is substantially differentiated from current catheter products that use the ineffective release of antibiotics or silver because we can covalently bind ("tether") AMPs uniformly onto catheter surfaces. Further, our research indicates our product will be a reimbursable, FDA Class II 510k medical device. Tethering of AMPs onto catheter surfaces is a non-traditional strategy of using AMPs, and because it allows maximized antimicrobial activity at the surface of the device where infection is most likely to occur. In our next value inflection point, we will prove that the use of our AMP coating product achieves the benchmarks of reducing 80% of CAUTIs, 200 patient hospital days, and tens of millions of dollars in *never event* expenses per 10,000 coated catheters sold. Competitively priced AMPs will provide maximum social and economic broader impact, shielding patients from devastating resistant infections and hospitals from *never event* costs and giving catheter manufacturers a new product that will differentiate them from their competitors.

IV. Market Opportunity and Addressable Patient Population

The AMProtection team validated the urinary catheter market pathway through market research and over 200 customer discovery interviews. *Interviews and market research indicated that the urinary catheter market has the highest addressable need* because of its large size, continued growth, high economic burdens on healthcare, lack of effective alternative treatments, and devastating impact on patient health. The global market for catheters in 2011 was $21.2 billion, and within this market, the cost to treat infection of central venous and urinary catheters is $3.5 billion. The cost of CAUTI care matches the urinary catheter market size at $1 billion per year in the U.S., which is split evenly between non-antimicrobial, uncoated catheters ($500,000,000) and antimicrobial, coated catheters ($500,000,000).

The occurrence of antibiotic resistance, high infection rate (5-6%) and changing healthcare policies have caused a considerable 5.4% CAGR for antimicrobial urinary catheters and an urgent need for better infection prevention. The Affordable Care Act changed the reimbursement landscape and shifted economic burdens from insurers to hospitals for several hospital acquired conditions. This included defining CAUTI as a *never event*, an unreimbursed hospital acquired condition that never should have occurred in the first place. The AMProtection opportunity comes through its ability to reduce *never events* with a broad-spectrum, biocompatible coating that will not promote antibiotic resistance. Many of our customer interviews suggested that antimicrobial resistance is a major market driver, since it is estimated to cost $100 trillion by 2050 if products that combat resistance are not developed. Preventing CAUTIs has become a substantial driver of economics in the urinary catheter market and in hospitals. Hospitals are no longer reimbursed for these infections, and bottom-performing hospitals may have their Medicare funding cut up to 2%. Therefore, purchasing decisions made by hospital materials acquisition departments for

antimicrobial catheters must consider the potential cost burden of CAUTIs versus overall savings on the device. This demand forces our target customers, catheter manufacturers, to differentiate their antimicrobial products and prove clinical benefit. The result is high competition, also a market driver. Unfortunately, current solutions are costly, toxic, and may promote antibiotic resistance.

The U.S. urinary catheter market is dominated by four major companies: Boston Scientific, Bard, Cook Medical and Teleflex. AMProtection will license, but the addressable market would depend on the licensee. For example, a license with Bard who owns 85% of the market share in both coated and uncoated catheter segments, may allow licensing revenue of $17-$42.5 million. A license with a smaller company such as Teleflex would allow for revenue of $1-$2.5 million. Revenues are expected to grow with widespread market adoption. (Estimations assume 50 million antimicrobial catheters sold per year in the U.S.).

V. Pricing and Reimbursement Strategies

Pricing

With the help of our mentors, we have estimated that our coating has a conservative cost of goods of approximately $5 per catheter, leading to a selling price of $10 per catheter to hospitals. This is competitive with products such as chlorhexidine-coated (ArrowEVOLUTION by Teleflex, $12) and silver-coated (Bacti-Guard by BARD, $14) catheters. As the prices of peptide manufacturing continue to decrease with improved synthesis technology and as we scale our product, we expect this manufacturing cost to be reduced.

Reimbursement

A key discovery during I-Corps and VentureWell E-Team was the vast influence of policy, regulatory and reimbursement climates on the urinary catheter market dynamics, which has created a favorable environment for the adoption of preventative (rather than reactive) antimicrobial products. The Affordable Care Act defining CAUTIs as unreimbursed has considerably interrupted the value chain. We have found predicate reimbursement codes of A4311/A4338 for urinary catheters, which according to our company interviews significantly increases the attractiveness of our technology.

VI. Commercialization Approach

Licensing Commercialization Strategy

It is not unusual for large medical device companies to try to gain market share through licensing and/or acquisition of smaller companies. The AMProtection licensing approach will be by field of use to urinary catheter manufactures for coating Foley urinary catheters. The impact and total addressable market depends on the licensee, but may reach as high as $42.5 million. A license will demonstrate technological validity and establish AMProtection as a *coating technology company*, rather than a catheter coating company.

Table 1: The AMProtection platform. Possible markets, sub-markets and customer segments.

Market	Sub-Markets	*Possible Customers*
Catheters	Urinary, central venous, PICCs	*Bard, Teleflex, Boston Scientific, Cook Medical*
Orthopedics	Knees, hips, pins, screws, plates	*J&J, Smith & Nephew, Zimmer, Stryker, Covidien*
Surgical Instruments	Scalpels, forceps, tweezers, sutures	*Covidien, Medtronic, Boston Scientific, Ethicon, VWR*
Packaging / Prep surfaces	Plastics, stainless steel	*Meat industry, distributors, caterers, Kimberly-Clarke*
Wound Care	Dressings, scaffolds	*Integra, J&J, Acelity, 3M*
Consumer Products	Toys, paints, pools, textiles, piping	*Hasbro, Tracker, US Navy, Namco, construction*
Water Filtration	Membranes, Piping	*Millipore, water treatment*

Potential Partnerships

Potential strategic partnerships for the manufacturing, packaging, storage and distribution of our coating product on urinary catheters would be the large companies that currently dominate the urinary catheter market, BARD, Boston Scientific, Teleflex and Cook Medical. We are currently in discussions with R&D experts at BARD and Boston Scientific to determine the most valuable innovations in urinary catheters. Additional conversations are being held with representatives at BVI, Cantabria Laboratories, and BMG Pharmaceuticals.

Validation and Expansion of the Commercialization Strategy

We determined that a licensing strategy is the most valuable commercialization plan for our platform. The economic benefit of multiple revenue streams (for different indications) and the flexibility for AMProtection to maximize core competency in surface-tethered AMP technologies

will allow us to pursue other fields of use, taking true advantage of this technology as a platform (*list of possible markets,* **Table 1**). Each unique market and customer would require an adapted business model. AMProtection plans to expand its AMP coating patent portfolio by in-licensing other technologies developed by the co-founders at WPI for other medical applications.

There are other business models to bring this technology to market, such as pursuing a joint venture, becoming a catheter manufacturer or a merger or acquisition by a larger company. We are diligently evaluating each option, but retain licensing as the primary strategy. The acquisition model has been observed before in the catheter market and would provide an exit for potential investors. Teleflex acquired Semprus Biosciences in 2012 for $30 million for an anti-adhesive coating not yet commercialized, pre-large animal stage. Then in 2013, Bard acquired Rochester Medical for $262 million for a coated antimicrobial urinary catheter.

Regulatory Strategy

The anticipated regulatory pathway is a 510(k) Class II medical device FDA pathway for our coating product, based on several interviews with FDA experts and consultants, and a predicate silver-coated urinary catheter device. This is an attractive pathway due to the lower barrier to entry. Potential partners include our potential customers: Bard, Teleflex, Boston Scientific, and Cook Medical. There is a precedent in the urinary catheter market for mergers and/or acquisitions of startup technologies before or during FDA trials. Our regulatory consultant, who has filed over 200 510(k) submissions and our regulatory lawyer both agree with this pathway.

- Development of a full regulatory strategy: Mary McNamara, RAC (Partner, Alira Health)
- Preparation for pre-IND and IND meetings with FDA: James Boiani, Epstein Becker Green

- Mentorship with clinical design: Dr. Douglas Waite, MD (CMO, Covidien Health), Dr. Jerrold Shapiro (CEO, Fem-Medical) and Dr. Yael Schwartz (CEO, Or-Genix Therapeutics)

We recently discussed this pathway with a former FDA regulatory officer in the department of urology. She directed us to product-specific guidance documents under 21 CFR 876.5130 for our urinary catheter product, guiding us to degradation ISO standards that are required for benchtop testing prior to meeting with FDA/CDRH/ODE/DRGUD/ULDB, Dr. Glenn Bell, Branch Chief.

Intellectual Property (IP) Strategy

For implementing our licensing approach, patents are our most important assets. Prior art searches reveal that there are no products currently on the market utilizing surface-bound AMPs to prevent infections. Other coatings for urinary catheters are primarily metal ion or chemical antiseptic-based. There are patents granted for other AMP coatings; however, based on our Freedom to Operate (FTO) analyses, our team and patent attorneys believe that we will have FTO within this landscape. Currently, systemic AMPs are not clinically utilized; however, AMPs for topical implications are in preclinical or clinical trials. Some of these therapies have failed Phase III clinical trials due to improper endpoints and trial design, such as Pexiganan, Omiganan (Microbiologix) and Iseganan (Intrabiotics). Other promising AMP therapies in Phase I or Phase II trials include NVB302 (Novacta), Plectasin (Sanofi) and Brilacidin (Cellcuetix). Given this IP and clinical landscape, we filed a patent (Application No. 62/205,206) and PCT (US2016/046792) with WPI's Office of Intellectual Property and Innovation and licensed patent attorneys at Greenberg Traurig (Boston, MA). Our patent protects the antimicrobial surface coating we have developed based on cysteine-modified AMP Chrysophsin-1. AMProtection has an exclusive

option to license the technology for 5% equity, non-diluted through the first round of funding, from WPI.

- *Patent status:* We recently received Office Action and have addressed reviewer concerns. The original patent claims are to be divided into separate inventions which are currently under review. We anticipate our most important claims to be granted based on phone discussions with the patent reviewer.
- *The most relevant prior art (and differences from our pending patent):*
    - Self-decontaminating coatings containing antimicrobial peptides (US8394762, 2013) [dissimilar AMP sequences and AMPs not covalently bound]
    - Medical devices and coatings with non-leaching antimicrobial peptides (US20070254006, 2007) [unpaid patent maintenance fees left the patent dead]
    - Anti-microbial compositions and devices and methods of using the same (US20090053278, 2009) [AMPs not covalently bound or modified in any way].

A current key activity for AMProtection is expanding its patent portfolio; our team is working with WPI to in-license additional patents based on other novel AMP coating technologies developed by our team at WPI that have broad medical applications.

An additional provisional patent application has been filed as of April 13th, 2018.

VII. Fundraising Goals, Milestones and Value Inflection Points

Fundraising Goals

We have raised $108,000 in non-dilutive funds through grants to perform customer interviews, determine product-market fit, understand our business model and value chain positioning, and to do preliminary benchtop testing of our technology. To commercialize the urinary catheter application, we estimate we need at total of $7-9 million and approximately 2-4 years until our first license deal (**Fig. 2**).

Our current dilutive/non-dilutive funding ask of $750,000 will take us from where we are currently at the benchtop stage through large animal studies, which is a significant value inflection point. A similar company to AMProtection, Semprus Biosciences, was acquired by Teleflex at this point along their development pathway. Within this seed round, there are 3 sub-milestones: (1) finishing benchtop testing and filing an IND, (2) small animal studies, and (3) large animal studies. The $750,000 raise will allow us to approach FDA for the start of Phase 1 (Safety) trails.

Following the seed round, we plan on raising a Series A of $3,000,000 dollars to achieve FDA Phase 1 (Safety), and FDA Phase 2 (efficacy) milestones, followed by a Series B of $6,000,000 for Phase 3 FDA trials through first sale. Each FDA trial represents a value inflection, which will raise our chance of licensing and partnering with one of our potential partners. Another similar company, Rochester Medical, was acquired by Cook Medical for a similar technology in 2013 for $262 million upon having FDA approval.

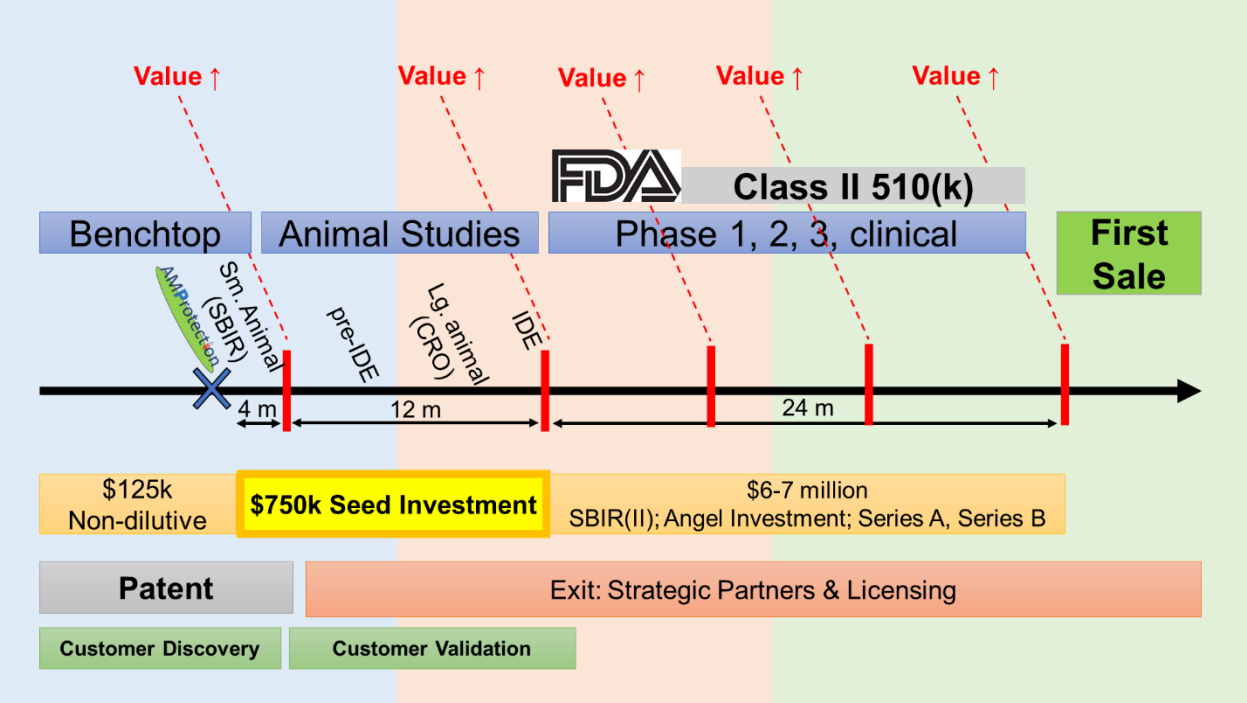


Figure 2: **Value inflections and general timeline.** The timeline, value inflection points, and estimated funds required for AMProtection to enter the urinary catheter market.

VIII. Financial Projections

Our financial projections (**Table 2**) are based on four major assumptions, a license with Bard, 50 million antimicrobial catheters sold per year, 10% market share in the first year, and a 10% royalty rate after FDA trials in year 2020. For the years 2021, and 2022 we assume 25% and 50% of Bards market share respectively. Our team also has estimated projections of licenses with the smaller players (e.g. Teleflex, Boston Scientific).

Table 2: Estimated revenue and costs through 2022.

	2018	**2019**	**2020**	**2021**	**2022**
Revenue	$0	$0	$2,975,000	$7,437,000	$14,875,000

We expect to be developing additional technologies during this pathway as soon as 2019 that will expand our platform and provide additional revenue streams.

IX. Compelling Preliminary Data

Current solutions for preventing catheter-associated urinary tract infections (CAUTIs) include antimicrobial coatings which hinder healing, promote inflammatory immune response, leach from the surface causing toxic side effects, and promote antibiotic resistance in bacteria. AMProtection has developed a coating for urinary catheters with the goal of improving upon these pitfalls to prevent CAUTIs, based on surface-tethered antimicrobial peptides (AMPs).

AMPs are proteins found in many species and are attractive alternatives due to their broad antimicrobial activity and low likelihood of developing resistance. AMPs have not become clinically relevant due mainly to their cytotoxicity and instability, which we believe our system overcomes. First, a competitive advantage of this coating technology is that the AMPs are covalently attached ("tethered") onto catheter polymers. We believe that attachment will reduce leaching, improve biocompatibility and increase stability while retaining antimicrobial efficacy. Second, we chose naturally derived AMP Chrysophsin-1 (CHY1) from the gills of the red sea bream. The marine derivation of CHY1 allows its improved salt tolerance and stability, and it has beneficial anti-inflammatory responses. Tethering of marine-derived AMPs had never been done prior to our recently published studies.

Table 4: **Minimum inhibitory concentrations of C-CHY1 demonstrate broad-spectrum antimicrobial activity.**

	Microbe	Published MIC for CHY1 (μM)	CHY1 (μM)	C-CHY1 (μM)	(1:1) C-CHY1:PEG (μM)*
Gm(-)	*E. coli* (HB101)	2.31	<0.625	7.92	7.92
	E. coli (B78)[a]	--	<0.625	12.5	15.83
	P. aeruginosa	>34.6	<0.625	4.58	8.33
Gm(+)	*S. epidermidis*[b]	0.432	<0.625	0.990	0.938
	S. aureus	2.31	<0.625	1.30	1.56
	Methicillin-resistant *S. aureus* (MRSA)	2.16	<0.625	1.56	1.46
	Methicillin-, Gentamycin-Resistant *S. aureus* (M/GRSA)	2.16	<0.625	1.67	1.77
	B. subtilis[c]	0.25	<0.625	0.990	0.990

[a]Clinical and resistant *E. coli* isolate, [b]Biofilm former, [c]Strain R01792 donated by NSRDEC.

In preliminary studies, we explored the feasibility of tethering and efficacy of CHY1 onto various surfaces, but most importantly polyurethane and silicone polymers, primary polymers in catheters. We first modified CHY1 with a cysteine residue (called C-CHY1) to allow for covalent binding

onto a tether molecule. We found that CHY1 demonstrated minimum inhibitory concentrations (MICs) between 0.25-5.0 μM, and that its modification to C-CHY1 did not significantly alter its potency (C-CHY1 MICs between 1-10 μM) (**Table 4**). When C-CHY1 was conjugated (1:1 molar ratio) to the tether molecule (poly(ethylene glycol), PEG), its MICs were below 20 μM. Strains tested included resistant and biofilm-forming strains. Further, soluble C-CHY1 and 1:1 C-CHY1:PEG were not cytotoxic to fibroblasts (ATCC CRL-2352) or uroepithelial cells (ATCC CRL-9520) up to 40 μM (data not shown). **These results suggest that modification of CHY1 to C-CHY1 and PEG conjugation did not significantly alter its antimicrobial efficacy, but created more biocompatible chimera for tethering applications.**

Proof-of-tethering was demonstrated using quartz-crystal microbalance with dissipation (QCM-D) on polyurethane polymers, the major polymer component of urinary catheters, but also on silicon dioxide (SiO_2), titanium dioxide (TiO_2) and gold (**Fig. 3A**). In QCM-D, large drops in frequency are proportional to mass addition. PEG was first introduced over a primary amine, then, the large decrease in frequency observed upon introduction of C-CHY1 to PEG indicates

successful binding (*indicated with arrows*). Using the well-established Sauerbrey relation, we calculate an areal mass of 1.9 µg/cm^2.

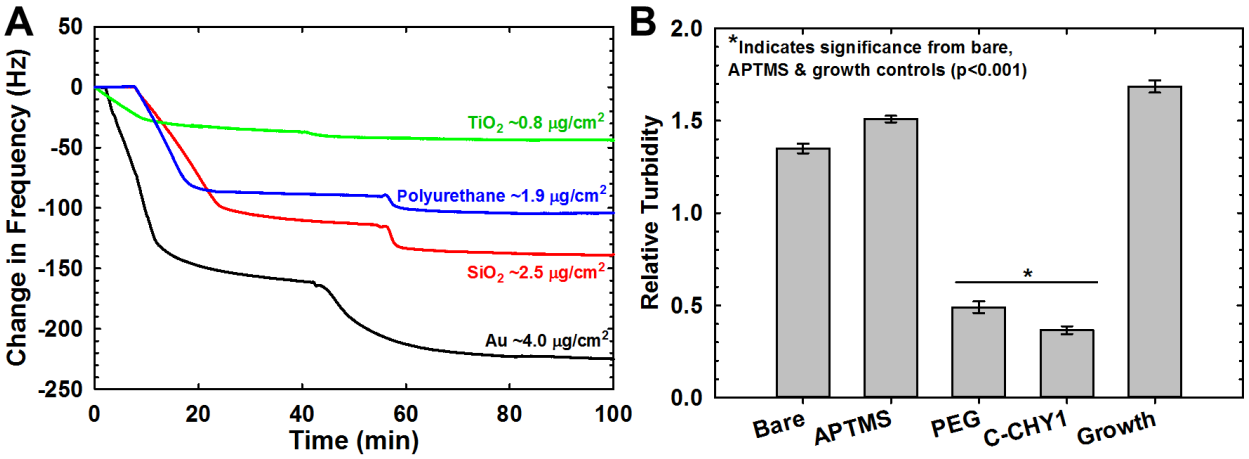


Figure 3: QCM-D proof of C-CHY1 tethering and antimicrobial activity. A, QCM-D profiles of change in frequency (Hz) vs. time (min), demonstrating binding (negative frequency) onto TiO_2 (green), polyurethane (blue), SiO_2 (red) and gold (black) substrates, and calculated average areal masses of attachment (µg/cm^2). **B,** Antimicrobial activity of tethered C-CHY1 coated on silicone polymer disks vs. *S. aureus*, demonstrated by a significant reduction of turbidity [*Bare = silicone polymer only; APTMS=primary amine only; PEG=tether only, C-CHY1=tethered peptide; growth=S. aureus in medium only*]. Values represent mean ± S.E., *n*=3.

We tested the antimicrobial activity of our tethered AMP coatings against a common pathogen in CAUTIs, *S. aureus*, using a procedure adapted from literature, where 5-mm diameter silicone disks were coated with C-CHY1, exposed to *S. aureus* for 16 hrs to allow bacterial attachment, well-rinsed with sterile medium, and then returned to sterile medium. After 24 hrs, bacterial growth was evaluated by reading absorbance at 590 nm (**Fig. 3B**). Turbidity of C-CHY1-coated disks, was significantly lower ($p<0.001$) compared to bare silicone, *S. aureus* only, and APTMS-only. As expected, PEG coatings also deterred *S. aureus* attachment, which has been demonstrated in previous studies. **This provides evidence that our coating binds to and is active on polymer surfaces similar to that of a catheter**, and demonstrates proof-of-concept that it will provide our anticipated 3-log reduction in bacterial growth benchmark on catheter surfaces.

We also explored the cytotoxicity of tethered C-CHY1 by tethering it onto sterile, ultra-low binding plates (polystyrene) and exposing fibroblast and uroepithelial cells for 16 hours (**Fig. 4**).

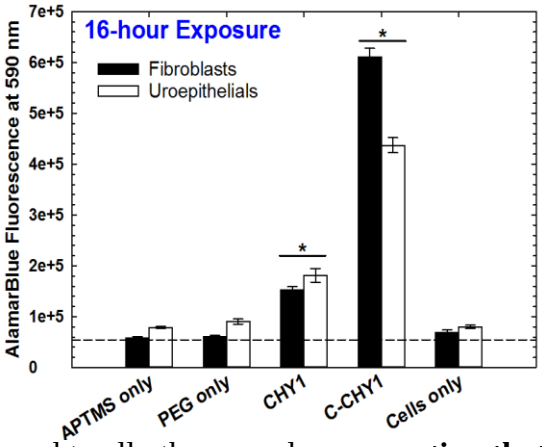


Figure 4: Cell viability after exposure to tethered AMPs. The fluorescent AlamarBlue® signal of human fibroblasts (black) and uroepithelial (white) cells after 16 hrs exposure to tethered C-CHY1 compared to APTMS and PEG coatings and soluble unmodified CHY1. The dotted line represents baseline AlamarBlue® signal. *p<0.001 of C-CHY1- and CHY1-tethered surfaces compared to all other samples. Values represent mean ± S.E.,n=3. Culture was done in ultra-low binding plates to ensure binding strictly to the coated surfaces, hence the low-viability "cells only"-control with no attachment.

C-CHY1 coatings demonstrated increased signal compared to all other samples, **suggesting that C-CHY1 not cytotoxic when tethered.**

X. An Overview of the Most Current Scientific Aims

Overall, our preliminary data demonstrates that modification of CHY1 for tethering (to C-CHY1) does not alter its antimicrobial activity, proof of C-CHY1 tethering and feasibility of antimicrobial activity on polymer surfaces similar to catheters, but also onto other surfaces as a platform and suggests that tethered C-CHY1 will be biocompatible.

Thus, the next experiments will be focused on developing an improved scientific understanding of our system in terms of important FDA studies and studies important for strategic partners. This includes demonstrating the coating's broad-spectrum antimicrobial ability, exploring its non-leaching character and long-term effectiveness and developing a strategy for sterilization and packaging methods for coated catheters. **We hypothesize** that C-CHY1 will retain its broad antimicrobial activity when exposed to polymicrobial infection, that tethering of C-CHY1 promotes long-term stability and continued antimicrobial efficacy on catheter surfaces over 30 days, and will be able to maintain antimicrobial activity after sterilization and dry packaging for 30 days.

The R&D plan will focus on the following scientific milestones:

1. **Determine the antimicrobial activity of tethered C-CHY1 coatings on Foley urinary catheters against polymicrobial infections.**
    a. Foley catheters will be cut into 1 cm segments under sterile conditions and dip-coated in 10% (v/v) 3-amino(propyltrimethoxysilane) (APTMS) in methanol for 20 min to create free primary amines. Segments will be rinsed in phosphate-buffered saline, pH 7.2 (PBS 7.2) and dip-coated in 100 µM (in PBS 7.2) sulfo-NHS-PEG-maleimide, molecular weight 7500 (PEG) "tether" for 30 minutes at 37°C. After another PBS 7.2 rinse, segments will be dip-coated in C-CHY1 at 10 µM (in PBS 7.2) at 37°C for 30

min. To test antimicrobial activity, segments will be rinsed in PBS 7.2 and placed in 24-well plates, and exposed to $1x10^6$ colony-forming units per mL (CFU/mL) of mixtures of different relevant microbes (bacteria and fungal strains) in Mueller Hinton Broth (MHB) and incubated at 37°C. After 1 hour, segments will be rinsed with buffer, stained with propidium iodide, and imaged at ex/em 493/636. Images will be analyzed with ImageJ (https://imagej.nih.gov/ij/) and percent bacterial mortality will be determined.

b. Benchmark: A 3-log reduction of viable microbial colonies, and improved local activity compared with both silver and uncoated segments.

2. Determine the long-term (30-day) degradation stability of tethered C-CHY1 coatings on Foley urinary catheters.

a. To determine if C-CHY1 leaches from the surface, 30-day degradation stability of C-CHY1 coatings on catheter segments will be tested using ISO 10993-13 protocols. The protocol allows calculation oxidative and chemical degradation of the coating using mass balances and Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), respectively. First, ATR-FTIR will be used to create a baseline chemical profile of bound C-CHY1 on catheter segments. Then, segments (C-CHY1, silver, and uncoated) will be dried with N_2 and mass will be measured using a balance sensitive to 0.01% of the total sample mass. After weighing, samples will be placed in the 3% (aq.) hydrogen peroxide (H_2O_2) at 70°C using a ratio of 1 g sample: 10 ml H_2O_2. At days 2 and 7 in H_2O_2, samples will be collected, rinsed with water, and dried with N_2. Dried samples will be weighed again and a mass balance will be calculated to determine

oxidative degradation. After the final weighing, ATR-FTIR will be performed on each sample *and* surrounding supernatant. ATR-FTIR chemical profiles at the beginning and end of the experiment will be compared to determine chemical changes of the coating and breakdown products due to degradation.

b. Benchmark: To show stability, samples must demonstrate a stable range <0.1% difference in mass, as per the standard.

3. Study the long-term (30-day) antimicrobial activity of tethered C-CHY1 coatings on Foley urinary catheters.

a. Foley catheter segments will be coated with peptide C-CHY1. At t=0, 24 hrs and weekly thereafter up to 30 days, segments (C-CHY1, silver, and uncoated, described in Objective 1) will be "challenged" with $1x10^6$ CFU/mL microbes in MHB *and* in simulated urine product, Surine™. At 1 hour after each challenge, segments will be rinsed with PBS 7.2, stained with propidium iodide, fluorescently imaged and analyzed as described in Objective 1. Between challenges, segments will be rinsed, sterilized with ethanol, and incubated in sterile growth medium or Surine™.

b. Benchmark: A 3-log reduction of viable microbial colonies for all bacterial challenges over time. This demonstrate improved local activity compared with both silver and uncoated segments.

4. Explore sterilization and dry packaging methods that will preserve the activity of coated Foley urinary catheters.

a. Coated catheters segments will be ethylene oxide sterilized and stored, dry, in autoclave bags. The long term antimicrobial activity and degradation will be studied as described in objectives 2 and 3 along different time points up to 3 months. If dry packaging does not maintain antimicrobial activity, then wet packaging methods will be explored.

a. Benchmark: The coated segments will maintain activity (3-log colony reduction of microbes) over the anticipated 3-month storage time.

Table 3: Timeline for Scientific Milestones

Milestone	Q1 (2018)			Q2 (2018)			Q3 (2018-2019)			Q4 (2019)		
	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	APR
M1: Polymicrobial activity												
M2: Stability (Antimicrobial)												
M3: Stability (Degradation)												
M4: Sterilization												

and packaging												
Other	**(a)**								**(b)**			**(c)**

Other activities **(from Table 3)**

(a) ***Mentorship with executive coaches, continue the interview process, serious discussions with strategic partners and potential customers.***

(b) ***Preparation for FDA visit***

(c) ***FDA visit and meeting (Rockville, MD)***

Additional scientific aims (pending SBIR) include small animal safety and efficacy studies. We expect to require $225,000 to implement the rest of the benchtop studies followed by an additional requirement for larger animal studies, in partnership with a CRO such as Tufts Veterinary or Charles River.

XI. Other Media

Videos…

- AMProtection Venture Forum 5 Minute Pitch: https://youtu.be/01ppASr23Jc
- AMProtection Two Minute Pitch: https://www.youtube.com/watch?v=gcQ1WsdzfDE
- AMProtection NSF I-Corps Technical video: https://youtu.be/XEUJ1o5QD_0

In the news…

- https://www.theventureforum.org/Resources/Documents/TVF%20Advantage%20April%202014.pdf
- https://www.theventureforum.org/the-venture-forum-in-the-news/3303374
- https://web.wpi.edu/Images/CMS/Business/VentureForum2016-NW.pdf
- https://www.wpi.edu/news/amprotection-shares-techsandbox-pitch-fest-prize
- https://www.wpi.edu/news/patenting-and-commercializing-wpi-research
- https://www.wpi.edu/news/announcements/amprotection-wins-techsandbox-pitch-fest-january-6th
- http://collegenewsupdates.com/todd-alexander-and-lindsay-lozeau-win-the-inaugural-hitchcock-innovation-prize/
- http://blogs.uml.edu/m2d2/2018/03/19/meet-an-m2d2-finalist-amprotection/
- http://www.telegram.com/news/20180408/competition-brings-out-best-in-central-mass-startups

At AMProtection, we ask the question, “Why wait until tomorrow to fight today’s infections?” With tomorrow’s antimicrobials, we can start reducing these infections, cutting hospital and patient cost burdens, but most importantly, saving lives – today.

Company Website

http://amprotection.us

Contacts

Lindsay D. Lozeau (Co-Founder)

Todd E. Alexander (Co-Founder)

Chapter 9 Conclusions and Future Directions

9.1 Conclusions

Antimicrobial resistance is a growing global threat and if left unchecked will cost over $100 trillion and tens of millions of lives unless novel antimicrobial solutions are developed [1, 2]. As alternative therapies, AMPs hold considerable promise in the fight against antimicrobial resistance due to their broad spectrum activity and unique mechanisms of action [3-10]. Due to the fact that their mechanism of action is typically non-specific against the bacterial membrane the likelihood of resistance is low; however, several technical and commercial barriers have kept them from widespread use in the clinic [3, 4, 6-8, 10-22]. Covalently tethering or binding the peptide to a surface is a strategy that can overcome these drawbacks, but careful selection of the AMP, tether chemistry, AMP density, and tether length play important roles in antimicrobial activity [3, 5, 11, 23-28]. We chose the broadly active, salt tolerant Chrysophsin-1 peptide as the active molecule for our antimicrobial surface coating [3, 29]. Chrysophsin-1 was modified at the N-terminal to allow for binding [3, 30].

In this thesis document, we aimed to develop a deeper technical and commercial understanding of a solution based on surface-bound AMP Chrysophsin-1. Using a modified version of Chrysophsin-1, we hypothesized that there is a relationship between the antimicrobial activity of tethered C-CHY1 and both spacer length and peptide surface density. Peptide density may be optimized via reaction temperature and salt concentration, increasing available surface binding sites. Supported lipid bilayers (SLBs) will allow for a better understanding of the mechanism of action (MOA) leading to better coating design, which will allow feasible development of a clinical business case for tethered AMPs.

First, we demonstrated that we could successfully bind C-CHY1and maintain antimicrobial activity and determined how tether length affects activity [3]. Using the QCM-D we examined binding of C-CHY1 onto three different molecular weight and length, polyethylene glycol (PEG

866, 2000, and 7500) molecules [3]. *E. coli* and *S. aureus* were used as model organisms to further our understanding of how tether length and grafting density affect peptide mechanisms, leading to antimicrobial activity [3]. A QCM-D allowed us to measure density and thickness simultaneously with antimicrobial activity [3, 31]. Each tether length had a different MOA [3]. We found that the longest tether PEG 7500 had the lowest density, but allowed for proper AMP orientation and its native pore-forming mechanism leading to the highest activity. The shortest tether, PEG 866, had the densest grafting comparatively, allowing for a non-native, potent ion displacement mechanism [32]. C-CHY1 peptides tethered with PEG 2000 could not wholly adopt either mechanism, by being neither sufficiently long nor dense, and thus were less effective. We demonstrated that tether length is an important parameter in AMP activity and can be used to alter the MOA. Nonetheless, density also plays a significant role in tethered C-CHY1 activity. We demonstrated that MOA of tethered AMPs can be rationally altered via spacer length. While there is disagreement in the literature, we suggest that longer spacers allow for a native mechanism of action and that studies that show activity for AMPs tethered to short spacers the mechanism of action is related to charge density verses action of the AMP itself.

In order to better understand how various factors affect the MOA we studied the soluble mechanisms of C-CHY1 [3, 5, 9, 24, 26-28, 33-38]. We used SLBs to determine the effects of lipid membrane components (LPS and LTA) and temperature (23^{o}C and 37^{o}C) had on the soluble mechanisms of C-CHY1 [39-43]. After using QCM-D to characterize the SLB thickness, density and fluidity with and without the LPS and LTA surface proteins, we monitored the dynamic interactions of C-CHY1 with each SLB. Despite having the similar MICs in solution against *E. coli* and *S. aureus*, the mechanistic steps leading to pore were different for Gram-negative and Gram-positive SLBs [3]. Based on our results C-CHY1 forms pores more easily in Gram-positive

SLBs, however these SLBs are limited in that they do not contain all of the membrane components of the actual bacteria [43-52]. For the Gram-negative membranes LPS did not have a significant effect on the MOA. LTA did have a significant effect on the MOA for Gram-positive membranes, with more peptide associating with the bilayer. Importantly, temperature altered the interaction of C-CHY1 with Gram-negative membranes likely due to the melting temperature of the lipids, which affected bilayer fluidity. SLBs can be used to screen other AMPs and inform clinical targets. This technique can be expanded to examine the effect of other surface proteins on MOA, however complexity should be balanced with the ability to interpret the data. For C-CHY1 specifically this suggests that it should be used for applications where Gram-positive bacteria are an issue.

From our previous study, peptide surface density, which is altered by grafting conditions, appeared to play a major role in altering tethered C-CHY1 activity. Thus using QCM-D, we investigated two different salt concentrations and five different temperatures (23^{o}C, 37^{o}C, 45^{o}C, 50^{o}C, and 55^{o}C) as grafting conditions and examined the effect on spacer density and peptide density of PEG 866 and 7500. We were able to achieve significantly higher density with PEG 7500 tethered C-CHY1. At 37^{o}C compared to 23^{o}C, C-CHY1 killed significantly higher amounts of Gram-positive *S. aureus*, but temperature had little effect against *E. coli*. Interestingly, we found that increasing density had no effect on activity for either bacteria on activity. This suggests that we either did not achieve a high enough C-CHY1 density to improve activity or that other means of improving activity need to be employed. Overall, with a better understanding of the soluble C-CHY1 mechanism of action, we can design a better surface coating to prevent infections.

Commercialization of medical devices requires understanding of the local market and regulatory conditions –which varies from country to country with many different regulatory bodies and differing levels of regulation— [53, 54], and antimicrobial resistance will disproportionately

affect countries in Africa and Asia [1]. Thus, for our study of foreign commercialization, we selected Nigeria because it is the largest country in Africa by population, there is an established regulatory system, and a large number of WPI IQPs that were available to be used as case studies [55, 56]. The IQPs examined the initial failure of a grassroots innovation developed by the Men on the Side of the Road (MSR), a non-profit organization designed to solve the issues of cost and sustainability of cooking fuels via compressed recycled paper blocks [57, 58]. A key take away from the Paper Block Project was the lack of understanding of the product market fit due to not engaging with the local community at the inception of the project [57, 59-63]. Additionally there was not consideration to the sustainability of the business model which poses additional challenges [59]. It is clear to improve the likelihood of success in any venture one must talk to and understand the needs of key stakeholders, which vary from country to country, an inherent feature in the iterative nature of the lean startup methodology [59, 63-66].

In order to find the product market fit for our technology we used both the lean startup method and the BMC as key tools to build a sustainable business model [63-67]. We performed over 250 customer interviews in order to find a market need, product-market fit, and test our hypotheses, and then used the BMC to develop a more formal business plan. After several BMC iterations, a business plan for coating Foley urinary catheters emerged as an initial revenue stream, chosen due to market need and well-defined regulatory pathway.

We also examined one case study to determine the benefits on entrepreneurial education for STEM graduate students [68]. We found that for WPI students the IGERT Ph.D. program had multiple benefits, including invention (6 patents and patent applications), innovation and venture creation through the STEM disciplines. Additionally, innovation and entrepreneurship become central to the university mission. This in in line with a growing body of evidence showing that

entrepreneurship programs add value to students and to their institutions [69-74]. Further follow up is needed as this is a single case study, but teaching entrepreneurship principles has a positive effect on student outcomes.

Overall, we were able to understand the most important parameters affecting the tethered cysteine modified chrysophsin-1 activity; including the effect of cysteine modification on the MOA, tether length and density [3]. Additionally we examined the effect of entrepreneurial education in graduate school, where we saw positive outcomes which included an increased impact of student projects [68].We also examined a case study in grassroots innovation in Namibia in order to better understand entrepreneurship in an international setting [59]. AMPs have faced many commercialization challenges, we sought to learn from past failures, and used the lean startup methodology along with the BMC to develop a sustainable business plan [8, 20, 63-67]. We also developed a business plan in order to take this coating to market and serve as a guide for other AMP based products.

9.2 Future Directions

AMPs are desirable due to their broad spectrum activity and low likelihood of resistance broad spectrum activity, however, they do have several drawbacks including cytotoxicity and low half-life in vivo [3, 10, 13-15, 17, 20, 27, 75-77]. A number of different strategies to overcome these challenges are available; cyclization, amino acid substitution, truncation, D-amino acids, non-canonical amino acids, hybridization, slow release, and covalently binding (tethering) AMPs [3, 6-9, 11, 13-16, 20, 25, 26, 28, 30, 33, 34, 45, 77-93]. In our study we tested tethering cysteine modified Chrysophsin-1 (C-CHY1) [3]. In solution C-CHY1 displayed low micro-molar activity against *S. aureus* and *E. coli*, Gram-positive and Gram-negative bacteria, respectively [3]. Unfortunately, while we were able to demonstrate significant activity against *S. aureus* we were unable to maintain significant activity against *E. coli* for the tethered C-CHY1 [3]. While our initial

results suggest a promising use of tethered C-CHY1 as an antimicrobial peptide, additional studies are required to increase and optimize the activity against a wider range of bacteria [8, 22, 23, 91, 94-100].

One possible solution is to rationally substitute amino acid residues of the C-CHY1 sequence [9, 34, 36, 83]. Hydrophobicity and charge are two major factors than can be altered rationally through substitution, however the RRRH end group of the peptide should not be modified, as it has been shown to be critical for activity [9, 33, 34, 36, 83, 101]. We have demonstrated these new analogs can be rapidly screened using our Gram-positive and Gram-negative SLB systems. SLBs can be used as a means of understanding their mechanism of action and the effect the amino acid substitution had, as well as look at concentration and temperature dependent effects [31, 42, 43, 82]. Furthermore, the QCM-D can be used to screen and determine an optimal tether length and density required for maximum antimicrobial activity [3, 31].

With the SLB work we were able to determine the MOA of C-CHY1 against Gram-positive and Gram-negative bacteria. However, our study was limited to one peptide concentration. It has been shown that the MOA of AMPs can be concentration dependent [39, 82] and preliminary findings for 10uM C-CHY1 shows that C-CHY1 interaction with the Gram-positive and Gram-negative bacteria is different compared to 5 µM, Supplemental Figures 1-5. The remaining data sets for the 10uM SLB interactions should be completed and additional concentrations tested. Based off of Lozeau *et al.* [82] and Wang *et al.* [43], we suggest testing concentrations of 0.25 µM, 0.5 µM, 1 µM, 2.5 µM and 20 µM, an adequate range to provide information on adsorption to sinking raft responses [51, 82].

Antimicrobial resistance has been an issue since the earliest days of antibiotic use [1, 2, 102, 103]. However, recent concern about antibiotic resistance has grown due to the dwindling

pipeline [1, 2, 104, 105]. Combination of traditional antibiotics with different mechanisms of action is one current approach to prevent the spread of resistance, another is to limit the use of last resort antibiotics so they are available when needed [2, 94, 106]. Nevertheless, the rise of multi-drug resistant (MDR) bacteria threatens the effectiveness of these approaches [104, 107]. It is important to remember these lessons as it is possible for bacteria to gain resistance to AMPs [7, 8, 108, 109]. Therefore for any antimicrobial coating it should be investigated to see if two different AMPs can be tethered to the same surface that have different MOA synergistic effects that may contribute better activity [22, 110-116]. Additionally AMPs can be combined with traditional antibiotics with synergistic effects [117, 118]. In future work we suggest the use of multiple AMPs in surface coatings, with synergy, and/or the use of multiple complimentary techniques.

Peptide density is an important parameter that affects the antimicrobial activity of tethered AMP systems [3, 24, 30, 91, 92, 119]. The use of QCM-D limited the maximum working temperature we could achieve (55^{o}C), which limited the density of PEG we could bind onto the surface [120-125]. We recommend testing density formation at higher temperatures, up to 90^{o}C, which could increase PEG density and thus higher AMP density leading to better activity [120, 121]. Additionally, we recommend examining additional substrates (e.g. titanium and polyurethane) to increase C-CHY1 density [14, 26, 28, 126]. Density and substrate composition plays an important role in AMP activity, as our findings on silicone coated polyurethane Foley catheters show significantly improved antimicrobial activity (data not shown).

In addition to understanding the important parameters the effect tethered C-CHY1 activity and MOA we examined a business case for this coating. This was due to the entrepreneurial training we received which allowed us envision broader societal impacts for our work. Therefore examined the benefits of teaching entrepreneurship to STEM graduate students [68]. Our results

are based on a single case study, hence we recommend conducting an adequate sample-size that can further explore the benefits of entrepreneurship training on student outcomes measured by both qualitative and quantitative outcomes. These outcomes include the students feeling towards entrepreneurship and their confidence in being an entrepreneur. Examples of quantitative outcomes include patent applications, grants such as SBIRs and start-ups. There is a growing body of evidence on the benefits of having entrepreneurship training for STEM students [71-74].

References

1. O'Neill, J., *Antimicrobial resistance: tackling a crisis for the health and wealth of nations.* Rev. Antimicrob. Resist, 2014. **20**: p. 1-16.
2. Centers for Disease Control and Prevention, *Antibiotic resistance threats in the United States, 2013. Atlanta: CDC; 2013*. 2014.
3. Lozeau, L.D., T.E. Alexander, and T.A. Camesano, *Proposed mechanisms of tethered antimicrobial peptide chrysophsin-1 as a function of tether length using QCM-D.* The Journal of Physical Chemistry B, 2015. **119**(41): p. 13142-13151.
4. Jenssen, H., P. Hamill, and R.E.J.C.m.r. Hancock, *Peptide antimicrobial agents.* 2006. **19**(3): p. 491-511.
5. Hilpert, K., et al., *Screening and characterization of surface-tethered cationic peptides for antimicrobial activity.* Chemistry & biology, 2009. **16**(1): p. 58-69.
6. Reddy, K., R. Yedery, and C. Aranha, *Antimicrobial peptides: premises and promises.* International journal of antimicrobial agents, 2004. **24**(6): p. 536-547.
7. Wimley, W.C. and K. Hristova, *Antimicrobial Peptides: Successes, Challenges and Unanswered Questions.* The Journal of Membrane Biology, 2011. **239**(1): p. 27-34.
8. Costa, F., et al., *Clinical Application of AMPs*, in *Antimicrobial Peptides: Basics for Clinical Application*, K. Matsuzaki, Editor. 2019, Springer Singapore: Singapore. p. 281-298.
9. Mishra, B., et al., *Host defense antimicrobial peptides as antibiotics: design and application strategies.* Current Opinion in Chemical Biology, 2017. **38**: p. 87-96.
10. Eckert, R., *Road to clinical efficacy: challenges and novel strategies for antimicrobial peptide development.* Future microbiology, 2011. **6**(6): p. 635-651.

11. Lozeau, L.D., et al., *Collagen tethering of synthetic human antimicrobial peptides cathelicidin LL37 and its effects on antimicrobial activity and cytotoxicity.* 2017. **52**: p. 9-20.

12. Rydlo, T., J. Miltz, and A. Mor, *Eukaryotic Antimicrobial Peptides: Promises and Premises in Food Safety.* Journal of Food Science, 2006. **71**(9): p. R125-R135.

13. Marr, A.K., W.J. Gooderham, and R.E. Hancock, *Antibacterial peptides for therapeutic use: obstacles and realistic outlook.* Current opinion in pharmacology, 2006. **6**(5): p. 468-472.

14. Gao, G., et al., *Antibacterial surfaces based on polymer brushes: investigation on the influence of brush properties on antimicrobial peptide immobilization and antimicrobial activity.* Biomacromolecules, 2011. **12**(10): p. 3715-3727.

15. Hancock, R.E. and H.-G. Sahl, *Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies.* Nature biotechnology, 2006. **24**(12): p. 1551.

16. Gao, G., et al., *Biomembrane Interactions Reveal the Mechanism of Action of Surface-Immobilized Host Defense IDR-1010 Peptide.* Chemistry & Biology, 2012. **19**(2): p. 199-209.

17. Hancock, R.E. and R.J.T.i.b. Lehrer, *Cationic peptides: a new source of antibiotics.* 1998. **16**(2): p. 82-88.

18. Hancock, R.E.J.T.L.i.d., *Cationic peptides: effectors in innate immunity and novel antimicrobials.* 2001. **1**(3): p. 156-164.

19. Hancock, R.E. and G.J.T.i.m. Diamond, *The role of cationic antimicrobial peptides in innate host defences.* 2000. **8**(9): p. 402-410.

20. Zasloff, M., *Antimicrobial Peptides: Do They Have a Future as Therapeutics?* 2016. p. 147-154.

21. The PEW Charitable Trusts. *Antibiotics Currently in Global Clinical Development*. 2019 March 4, 2019 [cited 2019 June 24th]; Available from: https://www.pewtrusts.org/en/research-and-analysis/data-visualizations/2014/antibiotics-currently-in-clinical-development.

22. Tobin, E.J., *Recent coating developments for combination devices in orthopedic and dental applications: A literature review.* Advanced Drug Delivery Reviews, 2017. **112**: p. 88-100.

23. Yu, K., et al., *Anti-adhesive antimicrobial peptide coating prevents catheter associated infection in a mouse urinary infection model.* Biomaterials, 2017. **116**: p. 69-81.

24. Bagheri, M., *Cationic Antimicrobial Peptides: Thermodynamic Characterization of Peptide-Lipid Interactions and Biological Efficacy of Surface-Tethered Peptides*. 2010, Freie Universität Berlin.

25. Li, Y., et al., *Effects of peptide immobilization sites on the structure and activity of surface-tethered antimicrobial peptides.* The Journal of Physical Chemistry C, 2015. **119**(13): p. 7146-7155.

26. Onaizi, S.A. and S.S. Leong, *Tethering antimicrobial peptides: current status and potential challenges.* Biotechnology advances, 2011. **29**(1): p. 67-74.

27. Haynie, S.L., G.A. Crum, and B.A. Doele, *Antimicrobial activities of amphiphilic peptides covalently bonded to a water-insoluble resin.* Antimicrobial Agents and Chemotherapy, 1995. **39**(2): p. 301-307.

28. Costa, F., et al., *Covalent immobilization of antimicrobial peptides (AMPs) onto biomaterial surfaces.* Acta biomaterialia, 2011. **7**(4): p. 1431-1440.

29. Iijima, N., et al., *Purification and characterization of three isoforms of chrysophsin, a novel antimicrobial peptide in the gills of the red sea bream, Chrysophrys major.* European Journal of Biochemistry, 2003. **270**(4): p. 675-686.

30. Ivanov, I.E., et al., *Creating Antibacterial Surfaces with the Peptide Chrysophsin-1.* ACS Applied Materials & Interfaces, 2012. **4**(11): p. 5891-5897.

31. Alexander, T.E., L.D. Lozeau, and T.A. Camesano, *QCM-D Characterization of Time-Dependence of Bacterial Adhesion.* The Cell Surface, 2019: p. 100024.

32. Kügler, R., O. Bouloussa, and F. Rondelez, *Evidence of a charge-density threshold for optimum efficiency of biocidal cationic surfaces.* Microbiology, 2005. **151**(5): p. 1341-1348.

33. Hicks, R.P., et al., *De novo design of selective antibiotic peptides by incorporation of unnatural amino acids.* Journal of medicinal chemistry, 2007. **50**(13): p. 3026-3036.

34. Han, H.M., R. Gopal, and Y. Park, *Design and membrane-disruption mechanism of charge-enriched AMPs exhibiting cell selectivity, high-salt resistance, and anti-biofilm properties.* Amino Acids, 2016. **48**(2): p. 505-522.

35. Tripathi, A.K., et al., *Identification of GXXXXG motif in Chrysophsin-1 and its implication in the design of analogs with cell-selective antimicrobial and anti-endotoxin activities.* Scientific reports, 2017. **7**(1): p. 3384.

36. Loose, C., et al., *A linguistic model for the rational design of antimicrobial peptides.* Nature, 2006. **443**(7113): p. 867.

37. Deslouches, B., et al., *Rational design of engineered cationic antimicrobial peptides consisting exclusively of arginine and tryptophan, and their activity against multidrug-resistant pathogens.* Antimicrobial agents and chemotherapy, 2013. **57**(6): p. 2511-2521.

38. Chen, Y., et al., *Rational design of α-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index.* Journal of biological chemistry, 2005. **280**(13): p. 12316-12329.

39. Lozeau, L.D., et al., *Concentration-Dependent, Membrane-Selective Activity of Human LL37 Peptides Modified with Collagen Binding Domain Sequences.* Biomacromolecules, 2018. **19**(12): p. 4513-4523.

40. Lozeau, L.D., et al., *A QCM-D study of the concentration-and time-dependent interactions of human LL37 with model mammalian lipid bilayers.* 2018. **167**: p. 229-238.

41. Wang, K.F., R. Nagarajan, and T.A. Camesano, *Antimicrobial peptide alamethicin insertion into lipid bilayer: a QCM-D exploration.* Colloids and Surfaces B: Biointerfaces, 2014. **116**: p. 472-481.

42. Wang, K.F., et al., *Characterization of supported lipid bilayer disruption by chrysophsin-3 using QCM-D.* The Journal of Physical Chemistry B, 2011. **115**(51): p. 15228-15235.

43. Wang, K.F., R. Nagarajan, and T.A. Camesano, *Differentiating antimicrobial peptides interacting with lipid bilayer: molecular signatures derived from quartz crystal microbalance with dissipation monitoring.* Biophysical chemistry, 2015. **196**: p. 53-67.

44. Kamaloo, E., *Supported lipid bilayer interactions with nanoparticles, peptides and polymers.* 2018.

45. Torcato, I.M., et al., *Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2013. **1828**(3): p. 944-955.

46. Michel, J.-P., et al., *Disruption of Asymmetric Lipid Bilayer Models Mimicking the Outer Membrane of Gram-Negative Bacteria by an Active Plasticin.* Langmuir, 2017. **33**(41): p. 11028-11039.

47. Lind, T.K., et al., *Formation and characterization of supported lipid bilayers composed of hydrogenated and deuterated Escherichia coli lipids.* PLoS One, 2015. **10**(12): p. e0144671.

48. Malanovic, N. and K. Lohner, *Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2016. **1858**(5): p. 936-946.

49. Sundh, M., S. Svedhem, and D.S. Sutherland, *Influence of phase separating lipids on supported lipid bilayer formation at SiO 2 surfaces.* Physical Chemistry Chemical Physics, 2010. **12**(2): p. 453-460.

50. Hardy, G.J., R. Nayak, and S. Zauscher, *Model cell membranes: Techniques to form complex biomimetic supported lipid bilayers via vesicle fusion.* Current opinion in colloid & interface science, 2013. **18**(5): p. 448-458.

51. Arouri, A., M. Dathe, and A. Blume, *Peptide induced demixing in PG/PE lipid mixtures: A mechanism for the specificity of antimicrobial peptides towards bacterial membranes?* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2009. **1788**(3): p. 650-659.

52. Flynn, K.R., et al., *Real-time quartz crystal microbalance monitoring of free docosahexaenoic acid interactions with supported lipid bilayers.* Langmuir, 2016. **32**(45): p. 11717-11727.

53. Brolin, S., *Global Regulatory Requirements for Medical Devices*. 2008, Akademin för hållbar samhälls-och teknikutveckling.

54. Kaushik, D., et al., *Regulatory perspectives on medical device approval in global jurisdictions.* Journal of Generic Medicines, 2013. **10**(3-4): p. 159-171.

55. Ikeme, A.C., *Nigeria's clinical trial scene.* Applied Clinical Trials, 2008. **17**(6): p. 70.

56. De Maria, C., et al., *Safe innovation: On medical device legislation in Europe and Africa.* Health Policy and Technology, 2018. **7**(2): p. 156-165.

57. Woodyard, A.H., et al., *THE PAPER BLOCK PROJECT: IMPROVING THE DESIGN AND IDENTIFYING MARKET OPPORTUNITIES FOR A SUSTAINABLE COOKING FUEL IN KATUTURA, NAMIBIA.* 2014.

58. Domingue, E.C., et al., *SUSTAINABLE PAPER INSULATION FOR KAMBASHUS IN INFORMAL SETTLEMENTS OF NAMIBIA.* 2012.

59. Dana, L.-P., et al., *Success factors and challenges of grassroots innovations: Learning from failure*. 2019.

60. Ries, E., *The lean startup methodology.* The Lean Startup, 2012.

61. Ries, E., *The lean startup: How today's entrepreneurs use continuous innovation to create radically successful businesses*. 2011: Crown Books.

62. York, J., *Putting Lean Startup into Perspective: A Novel Approach for Discovering and Developing a Successful Business Model.* Arch Bus Adm Manag: ABAM-104. DOI, 2018. **10**.

63. Blank, S., *Why the lean start-up changes everything.* Harvard business review, 2013. **91**(5): p. 63-72.

64. Blank, S. and J. Euchner, *The Genesis and Future of Lean Startup: An Interview with Steve Blank.* Research-Technology Management, 2018. **61**(5): p. 15-21.

65. Blank, S. and B. Dorf, *The startup owner's manual: The step-by-step guide for building a great company*. 2012: BookBaby.

66. Blank, S., *What's A Startup? First Principles.* 2010: https://steveblank.com/.

67. Osterwalder, A. and Y. Pigneur, *Business model generation: a handbook for visionaries, game changers, and challengers*. 2010: John Wiley & Sons.

68. Alexander, T.E., Lozeau, Linsday D., Camesano, Terri A., Hoy, Frank, *Applying Theory to Practice: Measuring Learning Outcomes from a STEM Doctoral Program.* Portland International Center for Management of Engineering and Technology (PICMET), 2019.

69. Thursby, M.C., A.W. Fuller, and J. Thursby, *An Integrated Approach to Educating Professionals for Careers in Innovation.* Academy of Management Learning & Education, 2009. **8**(3): p. 389-405.

70. Ohland, M.W., et al., *The effect of an entrepreneurship program on GPA and retention.* Journal of Engineering Education, 2004. **93**(4): p. 293-301.

71. Yazdi, Y. and S. Acharya, *A New Model for Graduate Education and Innovation in Medical Technology.* Annals of Biomedical Engineering, 2013. **41**(9): p. 1822-1833.

72. Huang-Saad, A., J. Fay, and L. Sheridan, *Closing the divide: accelerating technology commercialization by catalyzing the university entrepreneurial ecosystem with I-Corps™.* The Journal of Technology Transfer, 2017. **42**(6): p. 1466-1486.

73. Manbachi, A., et al., *Starting a Medical Technology Venture as a Young Academic Innovator or Student Entrepreneur.* Annals of Biomedical Engineering, 2018. **46**(1): p. 1-13.

74. Åstebro, T., N. Bazzazian, and S. Braguinsky, *Startups by recent university graduates and their faculty: Implications for university entrepreneurship policy.* Research Policy, 2012. **41**(4): p. 663-677.

75. Glinel, K., et al., *Antibacterial surfaces developed from bio-inspired approaches.* Acta Biomaterialia, 2012. **8**(5): p. 1670-1684.

76. Zasloff, M., *Antimicrobial peptides of multicellular organisms.* nature, 2002. **415**(6870): p. 389.

77. Brogden, N.K. and K.A. Brogden, *Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals?* International journal of antimicrobial agents, 2011. **38**(3): p. 217-225.

78. Li, X., et al., *Antimicrobial functionalization of silicone surfaces with engineered short peptides having broad spectrum antimicrobial and salt-resistant properties.* Acta biomaterialia, 2014. **10**(1): p. 258-266.

79. Kazemzadeh-Narbat, M., et al., *Antimicrobial peptides on calcium phosphate-coated titanium for the prevention of implant-associated infections.* Biomaterials, 2010. **31**(36): p. 9519-9526.

80. Kumar, P., J.N. Kizhakkedathu, and S.K. Straus, *Antimicrobial Peptides: Diversity, Mechanism of Action and Strategies to Improve the Activity and Biocompatibility In Vivo.* Biomolecules, 2018. **8**(1): p. 4.

81. Chu, H.-L., et al., *Boosting Salt Resistance of Short Antimicrobial Peptides.* Antimicrobial Agents and Chemotherapy, 2013. **57**(8): p. 4050.

82. Lozeau, L.D., et al., *Concentration-Dependent, Membrane-Selective Activity of Human LL37 Peptides Modified with Collagen Binding Domain Sequences.* Biomacromolecules, 2018.

83. Matsuzaki, K., *Control of cell selectivity of antimicrobial peptides.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2009. **1788**(8): p. 1687-1692.

84. Shukla, A., et al., *Controlling the release of peptide antimicrobial agents from surfaces.* Biomaterials, 2010. **31**(8): p. 2348-2357.

85. Tam, J.P., Y.-A. Lu, and J.-L. Yang, *Correlations of cationic charges with salt sensitivity and microbial specificity of cystine-stabilized β-strand antimicrobial peptides.* Journal of Biological Chemistry, 2002. **277**(52): p. 50450-50456.

86. Deslouches, B., et al., *De novo generation of cationic antimicrobial peptides: influence of length and tryptophan substitution on antimicrobial activity.* Antimicrobial Agents and Chemotherapy, 2005. **49**(1): p. 316-322.

87. Uzarski, J.R., et al., *The effects of solution structure on the surface conformation and orientation of a cysteine-terminated antimicrobial peptide cecropin P1.* Colloids and Surfaces B: Biointerfaces, 2008. **67**(2): p. 157-165.

88. Pasupuleti, M., et al., *End-tagging of ultra-short antimicrobial peptides by W/F stretches to facilitate bacterial killing.* PLoS One, 2009. **4**(4): p. e5285.

89. Hedegaard, S.F., et al., *Fluorophore labeling of a cell-penetrating peptide significantly alters the mode and degree of biomembrane interaction.* Scientific reports, 2018. **8**(1): p. 6327.

90. Hilpert, K., *Identifying novel antimicrobial peptides with therapeutic potential against multidrug-resistant bacteria by using the SPOT synthesis.* Mini-Reviews in Organic Chemistry, 2011. **8**(2): p. 157-163.

91. Bagheri, M., M. Beyermann, and M. Dathe, *Immobilization reduces the activity of surface-bound cationic antimicrobial peptides with no influence upon the activity spectrum.* Antimicrobial agents and chemotherapy, 2009. **53**(3): p. 1132-1141.

92. Fosgerau, K. and T. Hoffmann, *Peptide therapeutics: current status and future directions.* Drug discovery today, 2015. **20**(1): p. 122-128.

93. Batoni, G., et al., *Use of antimicrobial peptides against microbial biofilms: advantages and limits.* Current medicinal chemistry, 2011. **18**(2): p. 256-279.

94. Li, B. and T.J. Webster, *Bacteria antibiotic resistance: New challenges and opportunities for implant-associated orthopedic infections.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2018. **36**(1): p. 22-32.

95. Pickard, R., et al., *Antimicrobial catheters for reduction of symptomatic urinary tract infection in adults requiring short-term catheterisation in hospital: a multicentre randomised controlled trial.* The lancet, 2012. **380**(9857): p. 1927-1935.

96. Monzillo, V., et al., *Chlorhexidine-silver sulfadiazine-impregnated central venous catheters: in vitro antibacterial activity and impact on bacterial adhesion.* 2012. **35**(2): p. 175.

97. Bonfill, X., et al., *Efficacy and safety of urinary catheters with silver alloy coating in patients with spinal cord injury: a multicentric pragmatic randomized controlled trial. The ESCALE trial.* The Spine Journal, 2017. **17**(11): p. 1650-1657.

98. Kennedy, E.H., M.T. Greene, and S.J.J.o.h.m. Saint, *Estimating hospital costs of catheter-associated urinary tract infection.* 2013. **8**(9): p. 519-522.

99. Noimark, S., et al., *The role of surfaces in catheter-associated infections.* Chemical Society Reviews, 2009. **38**(12): p. 3435-3448.

100. Control, C.f.D. and Prevention, *Urinary tract infection (catheter-associated urinary tract infection [CAUTI] and non-catheter-associated urinary tract infection [UTI]) and other urinary system infection [USI]) events.* Centers for Disease Control and Prevention, Atlanta, GA: http://www. cdc. gov/nhsn/PDFs/pscManual/7pscCAUTIcurrent. pdf, 2015.

101. Mason, A.J., et al., *Membrane interaction of chrysophsin-1, a histidine-rich antimicrobial peptide from red sea bream.* Biochemistry, 2007. **46**(51): p. 15175-15187.

102. Control, C.f.D. and Prevention, *Antibiotic resistance threats in the United States, 2013*. 2013: Centres for Disease Control and Prevention, US Department of Health and ….

103. Nizet, V., *Antimicrobial peptide resistance mechanisms of human bacterial pathogens.* Current issues in molecular biology, 2006. **8**(1): p. 11.

104. Davies, J. and D. Davies, *Origins and Evolution of Antibiotic Resistance.* Microbiology and Molecular Biology Reviews, 2010. **74**(3): p. 417.

105. Centers for Disease Control and Prevention. *State-Specific Supplement to the National Healthcare-Associated Infection Standardized Infection Ratio Report: July 2009 through December 2009*. 2009 March 2, 2011 [cited 2019 January 28]; Available from: https://www.cdc.gov/HAI/surveillance/reports/state-specific-hai-sir-luly-dec-2009.html.

106. Worthington, R.J. and C. Melander, *Combination approaches to combat multidrug-resistant bacteria.* Trends in biotechnology, 2013. **31**(3): p. 177-184.

107. Luepke, K.H., et al., *Past, present, and future of antibacterial economics: increasing bacterial resistance, limited antibiotic pipeline, and societal implications.* Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 2017. **37**(1): p. 71-84.

108. Nawrocki, L.K., K.E. Crispell, and M.S. McBride, *Antimicrobial Peptide Resistance Mechanisms of Gram-Positive Bacteria.* Antibiotics, 2014. **3**(4).

109. Andersson, D.I., D. Hughes, and J.Z. Kubicek-Sutherland, *Mechanisms and consequences of bacterial resistance to antimicrobial peptides.* Drug Resistance Updates, 2016. **26**: p. 43-57.

110. Cloutier, M., D. Mantovani, and F. Rosei, *Antibacterial Coatings: Challenges, Perspectives, and Opportunities.* Trends in Biotechnology, 2015. **33**(11): p. 637-652.

111. Banerjee, I., R.C. Pangule, and R.S. Kane, *Antifouling coatings: recent developments in the design of surfaces that prevent fouling by proteins, bacteria, and marine organisms.* Advanced materials, 2011. **23**(6): p. 690-718.

112. Salwiczek, M., et al., *Emerging rules for effective antimicrobial coatings.* Trends in Biotechnology, 2014. **32**(2): p. 82-90.

113. Graham, M.V. and N.C. Cady, *Nano and microscale topographies for the prevention of bacterial surface fouling.* Coatings, 2014. **4**(1): p. 37-59.

114. Khoo, X. and M.W. Grinstaff, *Novel infection-resistant surface coatings: a bioengineering approach.* Mrs Bulletin, 2011. **36**(5): p. 357-366.

115. Tiller, J.C., *Antimicrobial surfaces*, in *Bioactive surfaces*. 2010, Springer. p. 193-217.

116. Yu, Q., Z. Wu, and H. Chen, *Dual-function antibacterial surfaces for biomedical applications.* Acta biomaterialia, 2015. **16**: p. 1-13.

117. Issam, A.-A., et al., *Pharmacological synergism of bee venom and melittin with antibiotics and plant secondary metabolites against multi-drug resistant microbial pathogens.* Phytomedicine, 2015. **22**(2): p. 245-255.

118. Feng, Q., et al., *Functional synergy of α-helical antimicrobial peptides and traditional antibiotics against Gram-negative and Gram-positive bacteria in vitro and in vivo.* European Journal of Clinical Microbiology & Infectious Diseases, 2015. **34**(1): p. 197-204.

119. Hickok, N.J. and I.M. Shapiro, *Immobilized antibiotics to prevent orthopaedic implant infections.* Advanced drug delivery reviews, 2012. **64**(12): p. 1165-1176.

120. Malmsten, M., K. Emoto, and J.M. Van Alstine, *Effect of Chain Density on Inhibition of Protein Adsorption by Poly(ethylene glycol) Based Coatings.* Journal of Colloid and Interface Science, 1998. **202**(2): p. 507-517.

121. Emoto, K., J.M. Harris, and J.M. Van Alstine, *Grafting poly (ethylene glycol) epoxide to amino-derivatized quartz: effect of temperature and pH on grafting density.* Analytical chemistry, 1996. **68**(21): p. 3751-3757.

122. Zdyrko, B., S.K. Varshney, and I. Luzinov, *Effect of Molecular Weight on Synthesis and Surface Morphology of High-Density Poly(ethylene glycol) Grafted Layers.* Langmuir, 2004. **20**(16): p. 6727-6735.

123. Kingshott, P., H. Thissen, and H.J. Griesser, *Effects of cloud-point grafting, chain length, and density of PEG layers on competitive adsorption of ocular proteins.* Biomaterials, 2002. **23**(9): p. 2043-2056.

124. Yang, Z., J.A. Galloway, and H. Yu, *Protein interactions with poly (ethylene glycol) self-assembled monolayers on glass substrates: diffusion and adsorption.* Langmuir, 1999. **15**(24): p. 8405-8411.

125. Lin, Y., V. Hlady, and C.-G. Gölander, *The surface density gradient of grafted poly (ethylene glycol): Preparation, characterization and protein adsorption.* Colloids and surfaces. B, Biointerfaces, 1994. **3**(1-2): p. 49.

126. Rai, A., et al., *High-density antimicrobial peptide coating with broad activity and low cytotoxicity against human cells.* Acta Biomaterialia, 2016. **33**: p. 64-77.

Supplemental Figures

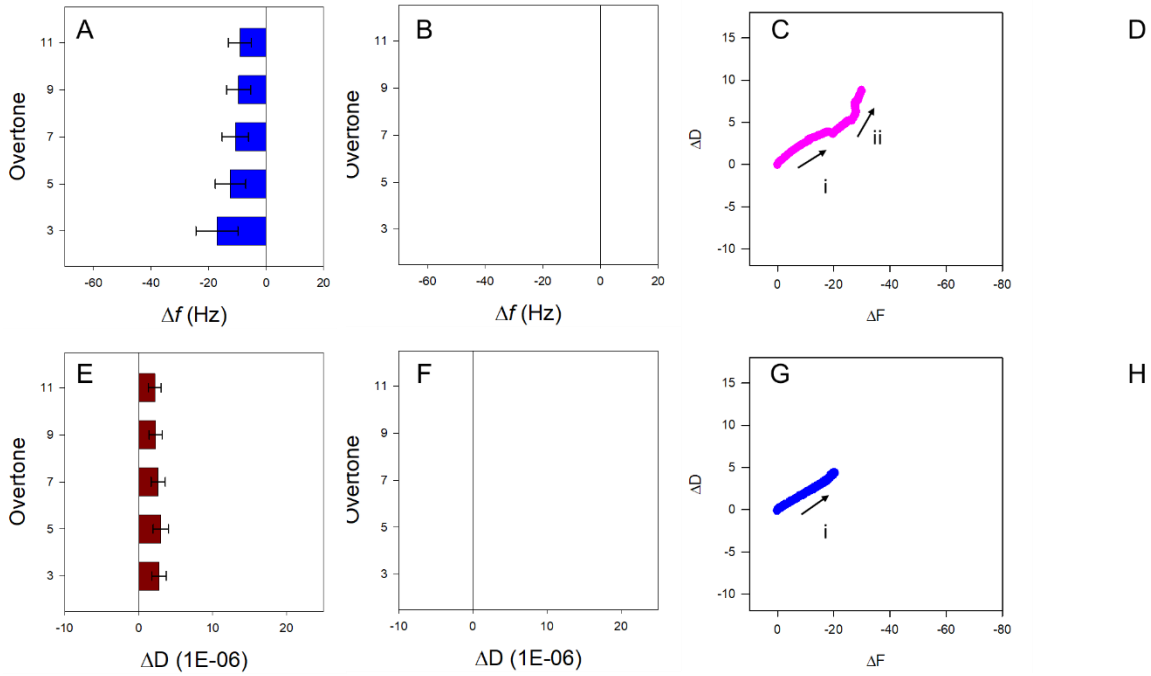


Figure 1: The overall and dynamic effect of LTA addition to SLBs at 23°C. Plots A, C, E and G are for PCPG bilayers at 23°C. Plots B, D, F and H are for PCPG+LTA bilayers at 23°C Overall Δ*f* and ΔD across the 3rd through 11th overtones for **A and E**, PCPG SLBs after C-CHY1 interaction and **B and F**, PCPG+LTA SLBs, after C-CHY1 interaction. ΔD vs. Δ*f* polar plots at the 3rd (C and D) and 11th (G and H) overtones for, PCPG and, PCPG+LTA SLBs respectively. All other overtones followed the same traces and are thus not shown.

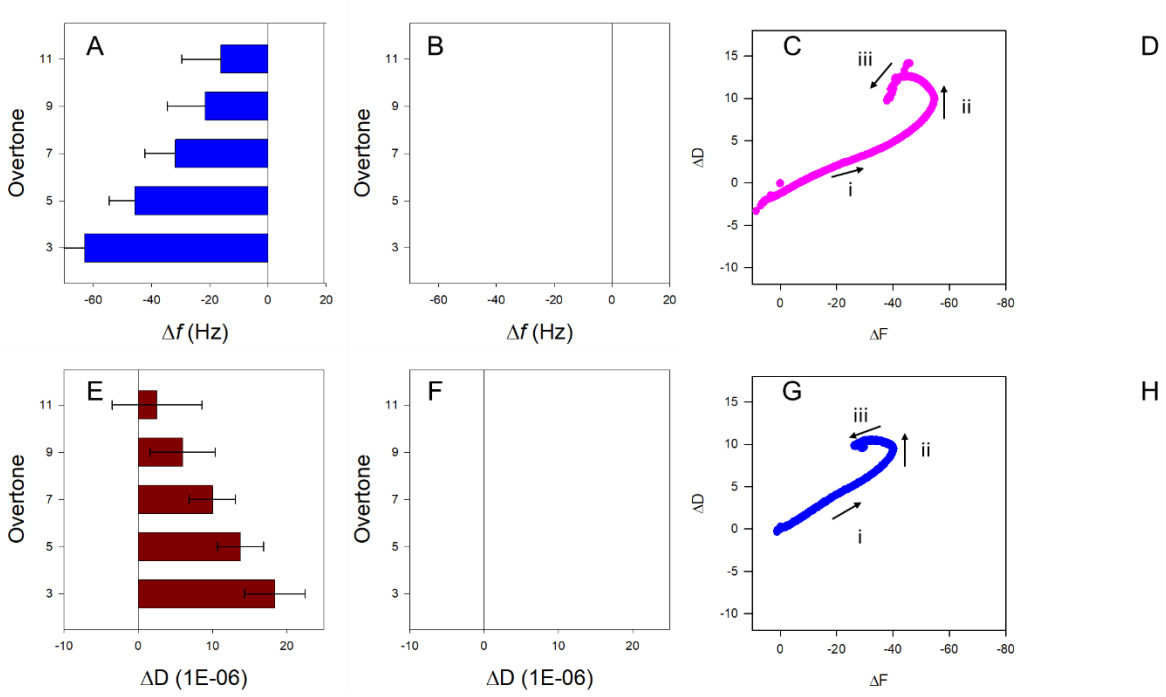


Figure 2: The effect of LTA addition to membranes at physiological temperature, 37^{o}C. Plots A, C, E and G are for PCPG bilayers at 23^{o}C. Plots B, D, F and H are for PCPG+LTA bilayers at 23^{o}C Overall Δ*f* and ΔD across the 3rd through 11th overtones for **A and E**, PC:PG SLBs after C-CHY1 interaction and **B and F**, PCPG+LTA SLBs, after C-CHY1 interaction. ΔD vs. Δ*f* polar plots at the 3rd (C and D) and 11th (G and H) overtones for, PCPG and, PCPG+LTA SLBs respectively. All other overtones followed the same traces and are thus not shown.

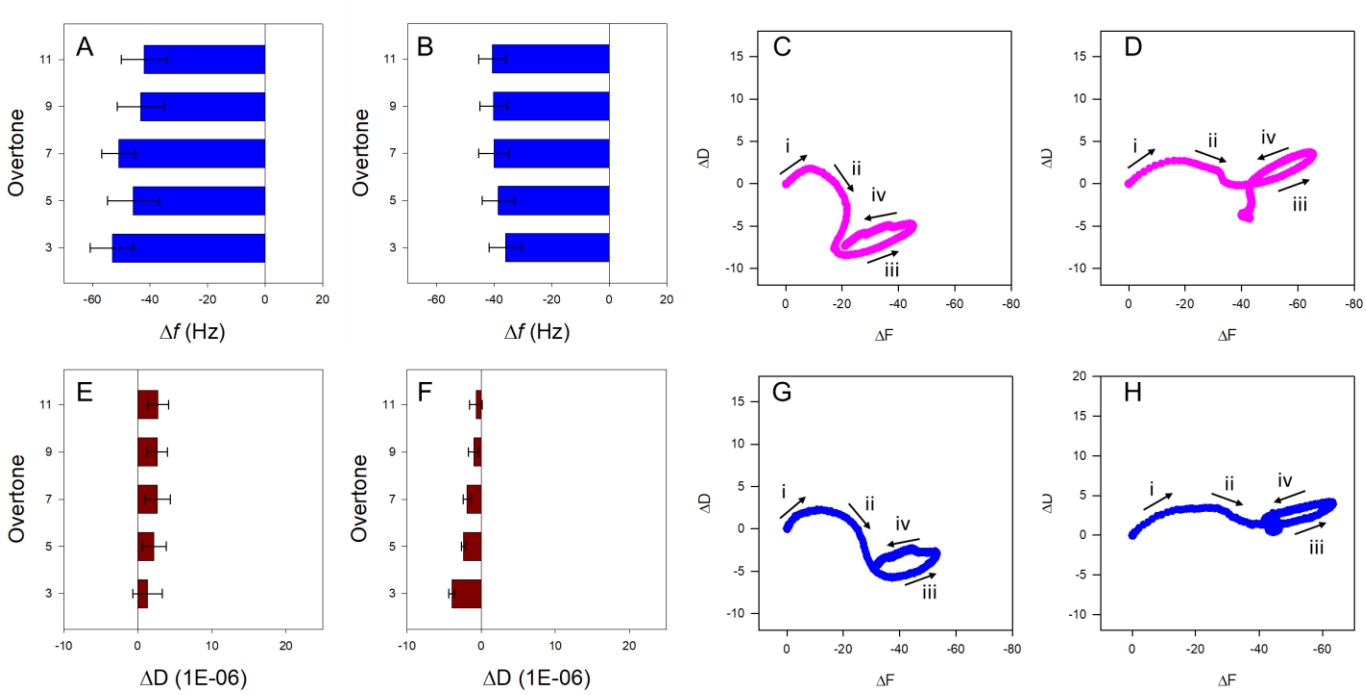


Figure 3: The effect of LPS addition to membranes at 23°C. Plots A, C, E and G are for PEPG bilayers at 23°C. Plots B, D, F and H are for PEPG+LPS bilayers at 23°C Overall Δf and ΔD across the 3rd through 11th overtones for A and E, PC:PG SLBs after C-CHY1 interaction and B and F, PEPG+LPS SLBs, after C-CHY1 interaction. ΔD vs. Δf polar plots at the 3rd (C and D) and 11th (G and H) overtones for, PEPG and, PEPG+LPS SLBs respectively. All other overtones followed the same traces and are thus not shown.

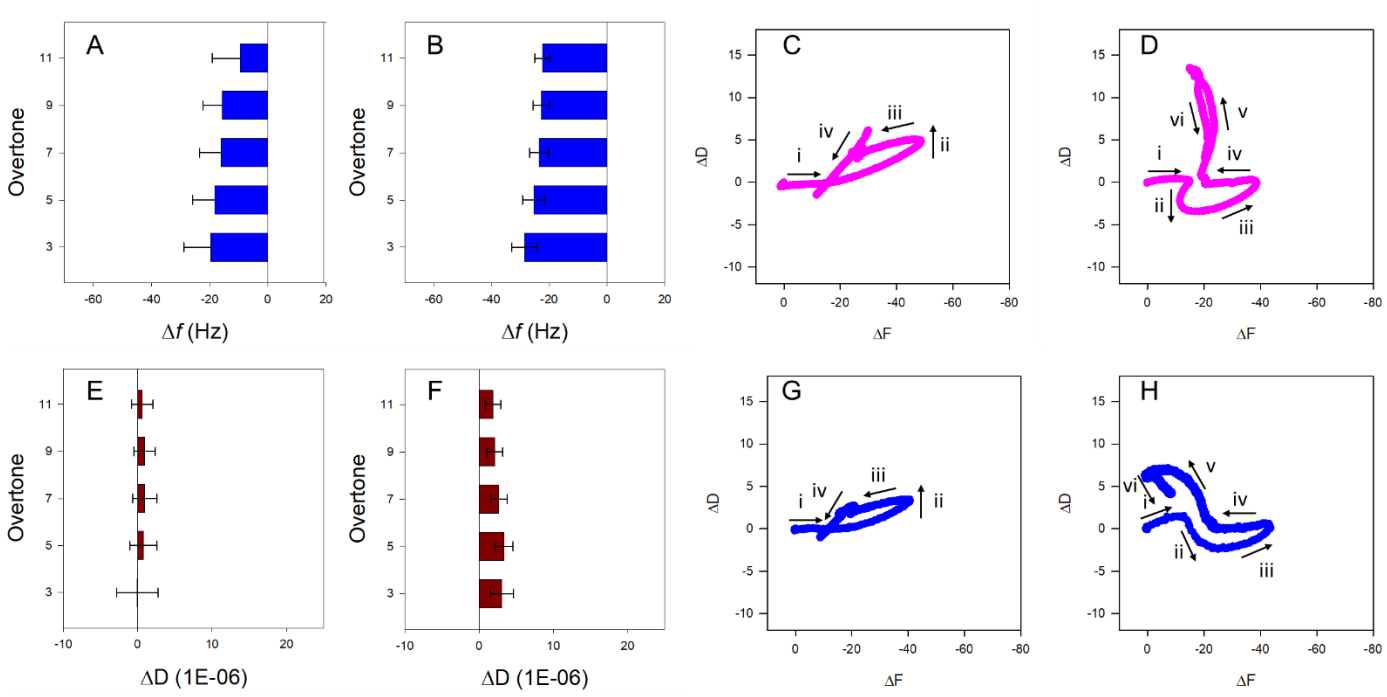


Figure 4: The effect of LPS addition to membranes at 37°C. Plots A, C, E and G are for PEPG bilayers at 23°C. Plots B, D, F and H are for PEPG+LPS bilayers at 23°C Overall Δf and ΔD across the 3rd through 11th overtones for A and E, PC:PG SLBs after C-CHY1 interaction and B and F, PEPG+LPS SLBs, after C-CHY1 interaction. ΔD vs. Δf polar plots at the 3rd (C and D) and 11th (G and H) overtones for, PEPG and, PEPG+LPS SLBs respectively. All other overtones followed the same traces and are thus not shown.

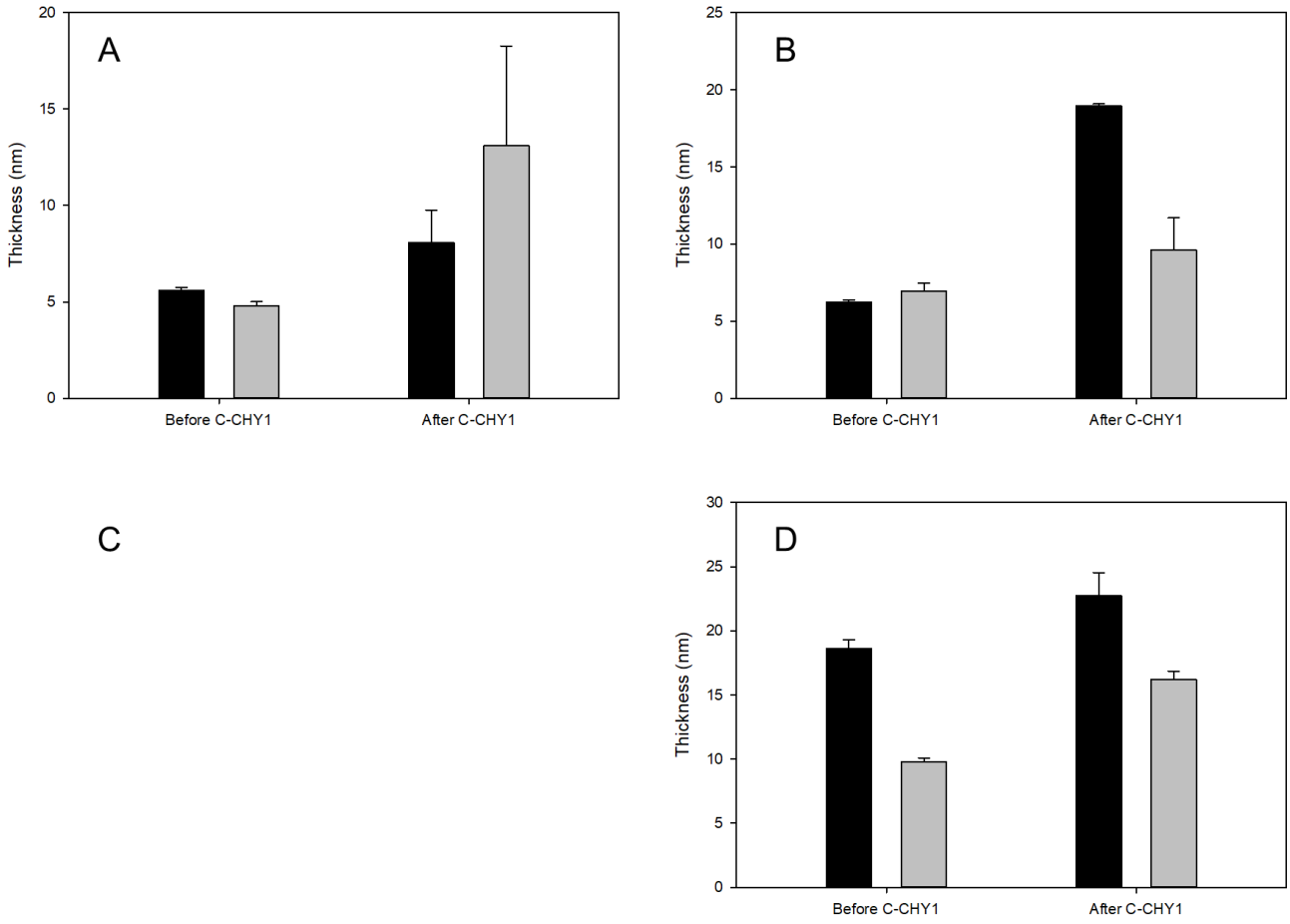


Figure 5: The overall bilayer thickness as a result of the addition of molecules to mimic Gram-positive and Gram-negative bacterial membranes, LTA and LPS. A) The thickness of PCPG bilayers before peptide interaction and after peptide interaction. B) The thickness of PEPG bilayers before peptide interaction and after peptide interaction. C) The thickness of PCPG+LTA bilayers before peptide interaction and after peptide interaction. D) The thickness of PEPG+LPS bilayers before peptide interaction and after peptide interaction. Statistical significance of $p<0.05$ was determined using a One Way Anova with a post hoc Tukey Test.