Investigating membrane morphological responses induced by antimicrobial lipids and correlations with biological activity

Yoon, Bo Kyeong

2018

Yoon, B. K. (2018). Investigating membrane morphological responses induced by antimicrobial lipids and correlations with biological activity. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/89428>

<https://doi.org/10.32657/10220/46250>

**INVESTIGATING MEMBRANE MORPHOLOGICAL
RESPONSES INDUCED BY ANTIMICROBIAL LIPIDS AND
CORRELATIONS WITH BIOLOGICAL ACTIVITY**

BO KYEONG YOON

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2018

**INVESTIGATING MEMBRANE MORPHOLOGICAL
RESPONSES INDUCED BY ANTIMICROBIAL LIPIDS AND
CORRELATIONS WITH BIOLOGICAL ACTIVITY**

BO KYEONG YOON

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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

2018

Statement of Originality

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

5 January 2018

.....
Date

.....
Bo Kyeong Yoon

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

5 January 2018

.....
Date

.....
Nam-Joon Cho

Authorship Attribution Statement

Chapter 4 is published as B.K. Yoon, J.A. Jackman, M.C. Kim, and N.J. Cho. Spectrum of Membrane Morphological Responses to Antibacterial Fatty Acids and Related Surfactants. *Langmuir* **31**, 10223–10232 (2015). DOI: 10.1021/acs.langmuir.5b02088.

The contributions of the co-authors are as follows:

- Professor Cho provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Dr. Jackman and Professor Cho.
- I designed the study, performed material synthesis, quartz crystal microbalance-dissipation (QCM-D) experiments, and antibacterial experiments, and analyzed all experimental data.
- Dr. Jackman advised on study design and data analysis.
- Dr. Kim conducted fluorescence microscopy experiments and assisted with data interpretation.

Chapter 5 is published as B.K. Yoon, J.A. Jackman, M.C. Kim, T.N. Sut and N.J. Cho. Correlating Membrane Morphological Responses with Micellar Aggregation Behavior of Capric Acid and Monocaprin. *Langmuir* **33**, 2750–2759 (2017). DOI: 10.1021/acs.langmuir.6b03944.

The contributions of the co-authors are as follows:

- Professor Cho provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Dr. Jackman and Professor Cho.
- I designed the study, performed material synthesis, fluorescence spectroscopy and QCM-D measurements, and analyzed all experimental data.
- Dr. Jackman advised on study design and data analysis.

- Dr. Kim conducted fluorescence microscopy experiments and assisted with data interpretation.
- Mr. Sut assisted with sample preparation and material synthesis for QCM-D measurements.

Chapter 6 is published as L.M. Kawakami¹, B.K. Yoon¹, J.A. Jackman, W. Knoll, P.S. Weiss, and N.J. Cho. Understanding How Sterols Regulate Membrane Remodeling in Supported Lipid Bilayers. *Langmuir* **33**, 14756–14765 (2017). DOI: 10.1021/acs.langmuir.7b03236.

(¹ denotes equal first authors)

The contributions of the co-authors are as follows:

- Professor Cho provided the initial project direction and edited the manuscript drafts.
- Professor Knoll and Professor Weiss advised on data interpretation and revision of manuscript drafts.
- I prepared the manuscript drafts with Ms. Kawakami. The manuscript was revised by Dr. Jackman, Professor Knoll, and Professor Weiss.
- Ms. Kawakami and I co-designed the study and jointly performed all experiments, including material synthesis and QCM-D measurements, and data analysis.
- Dr. Jackman advised on study design and data analysis.

5 January 2018

.....
Date

.....
Bo Kyeong Yoon

Abstract

Antimicrobial lipids such as fatty acids and monoglycerides are promising antibacterial agents that destabilize bacterial cell membranes, representing a new treatment approach that might overcome the challenges of antibiotic-resistant bacteria. To date, most studies on antimicrobial lipids have focused on evaluating inhibitory activity against target bacterium by indirect biological methods. While it is known that antimicrobial lipids act against bacterial cell membranes, it remains to be understood how antimicrobial lipids interact with phospholipid membranes. In this thesis, the objective is to develop a comprehensive physicochemical understanding of how fatty acids and monoglycerides interact with model phospholipid membranes, within the broader context of establishing an experimental framework based on supported lipid bilayers (SLBs) to correlate the molecular self-assembly of antimicrobial lipids with their corresponding biophysical and biological activities. The overall hypothesis of this thesis is that SLB platforms can provide a predictive model system to evaluate the potency and mechanism of action of antimicrobial lipids. To test this hypothesis, a fluid-phase, zwitterionic phospholipid SLB platform was established to investigate the membrane morphological responses induced by antimicrobial lipids, and surface-sensitive measurement techniques revealed that fatty acids promote tubule formation while monoglycerides induce membrane budding. Concentration-dependent trends in membrane-disruptive behavior were observed and correlated with the extent of inhibitory activity against *Staphylococcus aureus*. It was further determined that fatty acids induce tubule formation and increase membrane fluidity only at or above the corresponding critical micelle concentration (CMC) value, while monoglycerides exhibit dual-mode behavior by inducing membrane budding at or above CMC and promoting tubule formation at lower concentrations. In both cases, monoglycerides also increase membrane fluidity. By taking advantage of new SLB fabrication methods, it was also possible to study the interaction of fatty acids and monoglycerides with cholesterol-containing SLBs across different membrane phase states. Depending on the type of induced membrane morphological response, it was found that cholesterol either inhibited or promoted membrane remodeling in a manner consistent with how cholesterol affects the material properties of phospholipid/cholesterol

lipid bilayers. The experimental observations were rationalized by taking into account the chemical structure and self-assembly properties of antimicrobial lipids along with how these molecular characteristics influence membrane translocation and membrane strain in phospholipid membranes. In summary, the findings presented in this thesis demonstrate how SLB platforms can provide a predictive materials science tool for studying antimicrobial lipids, and offer a broadly applicable, integrated experimental approach to characterize the potency and mechanism of action of particular antimicrobial lipids.

Lay Summary

Conventional antibiotics that work against bacterial infections are becoming obsolete because bacteria are learning how to evade them and antibiotics no longer work. This issue is a major problem for public health. New classes of medicine that inhibit bacteria are needed, and antimicrobial lipids are a promising solution. While antimicrobial lipids have been studied for several decades, a key limiting factor to use them in medical applications has been the lack of experimental tools to understand the precise details of how antimicrobial lipids work. It is known that antimicrobial lipids damage the membrane coating surrounding bacterial cells, but how the damage occurs and how different antimicrobial lipids work are all outstanding questions. The objective of this thesis is to develop new experimental approaches to study antimicrobial lipids. Because bacterial cells are very complex, this new approach is based on simplified engineering platforms that mimic the membrane coating surrounding bacterial cells. Using this approach, it was discovered that two popular classes of antimicrobial lipid called fatty acids and monoglycerides interact with membrane coatings in different ways. Importantly, the effects of different amounts of the fatty acids and monoglycerides on the membrane coating was similar to the effects those same amounts had on bacterial cells. This finding indicated that the experimental approach provided a good predictive model for studying how antimicrobial lipids work against bacterial cells, with faster results and simpler experiments. By applying materials science and chemistry principles, it could be explained how the structure of antimicrobial lipids affects interactions with the membrane coating. In summary, the findings in this thesis demonstrate how engineering approaches can be utilized to study antimicrobial lipids and identify new potential drugs to treat bacterial infections.

Acknowledgements

I would like to express my sincere gratitude to Nanyang Technological University (NTU) and the International Graduate School in BioNanoTechnology (IGS BioNanoTech) program for giving me the once-in-a-lifetime opportunity to conduct my doctoral studies at NTU in Singapore and the University of Natural Resources and Life Sciences (BOKU) in Vienna, Austria. It has been such a wonderful journey to conduct scientific research across two continents.

I would also like to express my deepest gratitude to my supervisor, Professor Nam-Joon Cho, for all his support and patience throughout my doctoral studies, and I was so blessed to meet him and receive his encouragement to undertake doctoral studies. His passion for research and knowledge has constantly motivated me to continue my professional career in scientific research. Whenever I faced obstacles during my research, he gave me sincere advice and encouraged me to overcome the challenge. He is a great advisor on life matters as well as scientific research. I cannot imagine meeting a better supervisor for my doctoral studies and it would have been impossible to complete my thesis without his help throughout this whole journey.

I am grateful to my co-supervisor, Professor Wolfgang Knoll, for his continuous support during my research experience in Austria. It was an honor to have the opportunity to work with his group in Austria, and it has been truly wonderful to learn from him.

My sincere appreciation goes to Dr. Joshua Jackman. I was lucky to have such a good mentor like him who is a great researcher with limitless passion and dedicated commitment. He always carefully checked my research progress and gave insightful comments. It was most rewarding to have deep discussions with him about my research field, which helped me expand my scientific knowledge and become a better researcher.

Besides my advisors, special thanks are expressed to Professor Bo Liedberg from NTU and Professor Erik Reimhult from BOKU for their full support to complete the joint

doctoral program, IGS BioNanoTech. Additionally, I would like to thank the members of my Thesis Advisory Committee, Professor Wang Junling, Professor Tan Lay Poh, and Professor Liu Zheng, for their kind guidance throughout my doctoral studies.

During my time in the Engineering in Translational Science (ETS) group, I have met many talented group members and have had invaluable opportunities to work with them throughout my doctoral studies. I would like to first express my sincere thanks to Michael Potroz and Weibeng Ng, who were always keen to encourage me with positive energy throughout my studies. I am also very thankful to collaborate with Seong Oh Kim, Min Chul Kim, Jae Park, Soohyun Park, Dr. Ferhan Abdul Rahim, Saziye Yorulmaz, Elba Valle, and Tun Naw Sut. On a special note, I would like to express my sincere thanks to Natalia Mokrzecka with whom I closely worked. Her professional attitude inspired me to further develop, and it was enjoyable to work with her every moment. In addition, my special gratitude goes to Haram Jung for her sincere support and for helping me and our group. I also would like to thank to other group colleagues, including Ee-Lin Tan, Gaia Ferracci, and Hokyun Jinn.

Among overseas collaborators who visited ETS, I would like to thank Lisa Kawakami from UCLA, Kavous Kollahdouzan from Pomona College, and Danlin Li from Peking University for their dedicated passion for scientific research. It was a joy to work with all of you.

Last but not least, I would like to express my warmest and deepest gratitude to my beloved family: my grandmother, father, mother, older brother, and younger sister. Their endless prayers and love for me allowed me to sustain and overcome challenging moments during my doctoral studies in Singapore. Their support and belief in me was always constant and never wavered. Without their priceless support and love, this thesis would not have been possible.

**Dedicated to my family,
I love you dearly.**

Table of Contents

Abstract	i
Lay Summary	iii
Acknowledgements	v
Table of Contents	vii
Table Captions	xiii
Figure Captions	xv
Abbreviations	xxv
 Chapter 1 Introduction.....	 1
1.1 Hypothesis /Problem Statement	2
1.2 Objectives and Scope	6
1.3 Dissertation Overview	10
1.4 Findings and Outcomes/Originality	11
References.....	15
 Chapter 2 Literature Review	 19
2.1 Antimicrobial Lipids	20
2.1.1 Historical Origins	20
2.1.2 Classification	22
2.1.3 Spectrum of Antibacterial Activity	25

2.1.4	Mechanisms of Antimicrobial Activity	32
2.2	Experimental Approaches to Characterize Antimicrobial Lipids	37
2.2.1	Anti-Infective Evaluation of Bacterial Specimens	38
2.2.1.1	Growth Inhibition Assays	38
2.2.1.2	Infectivity Assays	42
2.2.1.3	Electron Microscopy	43
2.2.2	Biophysical Approaches with Model Membrane Platforms	47
2.2.2.1	Solution-Phase Liposomes	47
2.2.2.2	Giant Unilamellar Vesicle	52
2.2.2.3	Supported Lipid Bilayers	54
2.3	Outstanding Questions	59
2.3.1	Correlation between Biophysical and Biological Activities	59
2.3.2	Physicochemical Distinctions between Fatty Acids and Monoglycerides	60
	References	62
Chapter 3	Experimental Methodology	69
3.1	Rationale for the Selection of Materials and Experimental Techniques	70
3.2	Materials	72
3.2.1	Single-Chain Lipid Amphiphiles	72
3.2.2	Phospholipid Reagents	73
3.2.3	Buffer Reagents	74
3.2.4	Bacterial Cell Strains and Antibacterial Testing Reagents	74
3.3	Materials Synthesis	75
3.3.1	Preparation of Antimicrobial Lipid Solutions	75
3.3.2	Supported Lipid Bilayer Preparation	76

3.3.3	Bacterial Cell Culture	79
3.4	Experimental Techniques	81
3.4.1	Critical Micelle Concentration Determination Assay	81
3.4.2	Quartz Crystal Microbalance-Dissipation.....	86
3.4.3	Fluorescence Microscopy	91
3.4.4	Fluorescence Recovery After Photobleaching.....	94
3.4.5	Minimum Inhibitory Concentration (MIC) Testing.....	97
	References	101
Chapter 4	Spectrum of Membrane Morphological Responses	105
4.1	Introduction	106
4.2	Materials and Methods	109
4.2.1	Materials	109
4.2.2	Preparation of Antimicrobial Lipid Solutions.....	109
4.2.3	Quartz Crystal Microbalance-Dissipation (QCM-D) Experiments	110
4.2.4	Fluorescence Microscopy Experiments	111
4.2.5	Bacterial Cell Culture	112
4.2.6	Minimum Inhibitory Concentration (MIC) Testing.....	112
4.3	Results	113
4.3.1	Effect on Mass and Viscoelastic Properties of Supported Lipid Bilayers	113
4.3.1.1	Sodium Dodecyl Sulfate.....	113
4.3.1.2	Lauric Acid.....	115
4.3.1.3	Glycerol Monolaurate	116
4.3.2	Observation of Morphological Changes in Supported Lipid Bilayers	118
4.3.2.1	Sodium Dodecyl Sulfate.....	119

4.3.2.2	Lauric Acid.....	120
4.3.2.3	Glycerol Monolaurate	122
4.3.3	Microbicidal Activity against <i>S. aureus</i> Bacteria	124
4.4	Discussion	125
4.4.1	Supported Lipid Bilayer as an Experimental Platform	125
4.4.2	Physicochemical Basis for Morphological Changes.....	126
4.4.3	Role of Surfactant Concentration on Membrane Disruption	127
4.5	Conclusion.....	128
	References.....	130

Chapter 5 Correlating Membrane Morphological Responses with Micellar Aggregation133

5.1	Introduction	134
5.2	Materials and Methods.....	137
5.2.1	Materials	137
5.2.2	Preparation of Test Compound Solutions	137
5.2.3	Fluorescence Spectroscopy	138
5.2.4	Quartz Crystal Microbalance-Dissipation (QCM-D) Experiments	138
5.2.5	Fluorescence Microscopy	139
5.2.6	Fluorescence Recovery After Photobleaching (FRAP) Measurements.....	140
5.3	Results and Discussion.....	140
5.3.1	Determination of the Critical Micelle Concentration	140
5.3.2	Effect on Mass and Viscoelastic Properties of Supported Lipid Bilayers .	142
5.3.2.1	Capric Acid	144
5.3.2.2	Monocaprin	145

5.3.3	Observation of Morphological Changes in Supported Lipid Bilayers	147
5.3.3.1	Capric acid.....	148
5.3.3.2	Monocaprin	150
5.3.4	Effect on Lateral Lipid Diffusion	153
5.4	Conclusion.....	156
	References.....	157
Chapter 6	Understanding How Sterols Regulate Membrane Remodeling.....	161
6.1	Introduction	162
6.2	Materials and Methods	165
6.2.1	Reagents.....	165
6.2.2	Sample Preparation	166
6.2.3	Quartz Crystal Microbalance-Dissipation (QCM-D).....	166
6.3	Results and Discussion.....	167
6.3.1	Formation of Cholesterol-Rich Supported Lipid Bilayers.....	167
6.3.2	Evaluation of Membrane Morphological Responses	170
6.3.2.1	Sodium Dodecyl Sulfate.....	171
6.3.2.2	Lauric Acid.....	173
6.3.2.3	Glycerol Monolaurate	175
6.3.3	Influence of Cholesterol on Membrane Remodeling.....	176
6.4	Conclusion.....	179
	References.....	181
Chapter 7	Discussion and Future Work	185
7.1	Discussion and Concluding Remarks.....	186

7.2	Future Outlook	191
7.2.1	Investigation of Glycerol Monolaurate Equivalent with Ether Backbone.	191
7.2.2	Synergistic Mixtures of Fatty Acids and Monoglycerides	195
	References	199
	Appendix	201

Table Captions

Table 2.1 Antibacterial activity of fatty acids and monoglycerides against different bacterial strains.

Table 2.2 Selected MIC values of fatty acids against different bacteria.

Table 2.3 Selected MIC values of monoglycerides against different bacteria.

Table 2.4 Electron microscopy studies reporting how antibacterial fatty acids and monoglycerides affect bacterial cells.

Table 2.5 Investigation of fatty acid interactions with solution-phase liposomes.

Table 2.6 Interactions of antimicrobial lipids and related single-chain lipid amphiphiles with SLB platforms.

Table 4.1 MIC values for antimicrobial lipids against *S. aureus* bacteria. The MIC values are reported from a minimum of three independent experiments using a two-fold dilution protocol.

Table 5.1 Summary of diffusion coefficients measured for supported lipid bilayers before and after treatment with capric acid or monocaprin, as determined by FRAP measurements.

Figure Captions

Figure 2.1 Chemical structures of fatty acids and monoglycerides. Saturated fatty acids; Capric acid (C10:0), Lauric acid (C12:0), Monoglycerides; Monocaprin (MG C10:0), Glycerol monolaurate (MG C12:0), Unsaturated fatty acids: Oleic acid (C18:1), Elaidic acid (*trans*-C18:1), Polyunsaturated fatty acids; Linoleic acid (C18:2), Linolenic acid (C18:3). The pKa values of fatty acids are: Capric acid (~4.9, Ref. [15]); Lauric acid (~5.3, Ref. [16]); Oleic acid (~9.8, Ref. [17]); Elaidic acid (~9.9, Ref. [17]); Linoleic acid (~9.2, Ref. [17]); and α -Linolenic acid (~8.3, Ref. [17]). Cx:y is defined such that x is the number of carbons in the primary alkyl chain and y is the number of degrees of unsaturation.

Figure 2.2 Schematic representation of mechanisms behind the antibacterial activity of fatty acids and monoglycerides.

Figure 2.3 TEM micrographs show the effect of treating bacterial cells with fatty acids and monoglycerides. *L. monocytogenes* cells that are (A) untreated or (B) treated with 50 μ g/ml GML [38] and *C. perfringens* cells that are (C) untreated or (D) treated with 1 mg/ml lauric acid [47].

Figure 2.4 DLS measurements and complementary freeze-fracture electron micrographs measuring the interaction of fatty acids with SUVs and LUVs. DLS-measured size distribution curves of POPC lipid vesicle extruded with (A) 50 nm, (B) 100 nm diameter pore filters before (curve b) and after (curve c) oleate addition [84], and freeze-fracture micrographs of (C) preformed 180-nm diameter Egg PC vesicles and (D) Egg PC/oleate (1:1) vesicles. The scale bars are 200 nm [87].

Figure 2.5 Optical micrographs showing the morphological responses of POPC GUVs that occur upon treatment with 0.8 mM oleic acid solution [98].

Figure 2.6 Fluorescence micrographs depicting the morphological responses of SLBs. The morphological responses occurred after treatment with 50 μM lysophosphatidylcholine (LPC) in (A) 250 mM KCl and (B) 50 mM KCl [100], and (C) 200 μM docosahexaenoic acid (DHA) and corresponding (D) proposed mechanism of tubule formation [104].

Figure 3.1 Chemical structures of the tested antimicrobial lipids and related compounds used in this thesis.

Figure 3.2 Chemical structures of the tested phospholipids and cholesterol in this thesis.

Figure 3.3 Principle of solvent-assisted lipid self-assembly. Phospholipids are dissolved in pure isopropanol and exist as inverted micelles. A solvent-exchange step is conducted and the phase state of the phospholipids changes, yielding the bottom-up formation of an SLB on the solid support [23].

Figure 3.4 Fluorescence microscopy observation of cholesterol-rich SLBs with different cholesterol molar fractions, as prepared by the SALB method on glass surfaces [6].

Figure 3.5 Colony-forming unit (CFU) quantification result of *S. aureus* (ATCC 25923) cell culture at 20-times dilution for $\text{OD}_{600} = 0.1$. Agar plates containing between 25 and 250 cell colonies, which was the applicable range for plates with 10^3 dilution, were selected for CFU enumeration. A 20-fold diluted sample of *S. aureus* (ATCC 25923) cells with an original OD_{600} value of ~ 0.1 has a cell density of $\sim 2 \times 10^6$ CFU/ml. Noted that different streaking volumes of 10, 50, and 100 μl of diluent at each dilution factor were tested and based on similar results at all tested volumes, 10 μl was selected as the standard condition for all CFU quantification experiments.

Figure 3.6 Jablonski diagram depicting fluorescence excitation and emission steps [27].

Figure 3.7 Chemical structure of the fluorescence probes, pyrene and 1-pyrenecarboxaldehyde, respectively. 1-pyrenecarboxaldehyde was used to determine the CMC experiments in this thesis.

Figure 3.8 Fluorescence spectrum of 1-pyrenecarboxaldehyde in PBS solution upon excitation at 365.5 nm wavelength.

Figure 3.9 Determination of critical micelle concentration using the 1-pyrenecarboxaldehyde fluorescent probe. Peak wavelength is presented as a function of surfactant concentration in (A) water and (B) PBS. The PBS buffer [pH 7.2] consists of 1.54 mM KH_2PO_4 , 155.17 mM NaCl, and 2.71 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$.

Figure 3.10 Representative CMC determination for the GML compound by the 1-pyrenecarboxaldehyde fluorescent probe method. (A) Fluorescence spectrum shift as a function of GML concentration and (B) Peak wavelength shift as a function of GML concentration in PBS solution.

Figure 3.11 Schematic representation of monitoring the oscillation decay in QCM-D measurements. (A) AC voltage is applied between electrodes. When the AC voltage is turned off, oscillation decay occurs and representative decay patterns for a (B) rigid and (C) viscoelastic adsorbate.

Figure 3.12 Schematic representation of different model membrane platforms that are routinely studied in QCM-D applications, including adsorbed vesicle layer and supported lipid bilayer [40].

Figure 3.13 Methyl- β -cyclodextrin treatment of cholesterol-containing SLB platforms. (A) Fluorescence micrograph of cholesterol-enriched SLB after M β CD treatment. (B) Increase amount of cholesterol depletion after 1 mM M β CD treatment as a function of cholesterol fraction used in the SLB fabrication [6]

Figure 3.14 Schematic diagram of epifluorescence microscope setup [27].

Figure 3.15 Microscopic observation of (A) LPC treatment on SLBs containing 30 mol% dioleoylphosphatidic acid (DOPA), 69.5 mol% DOPC, and 0.5 mol % tail-labeled nitrobenzofuran-conjugated PC (tail-labeled NBD-PC) [18] and (B) DHA treatment on SLBs composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphocholine (POPC) with additional 0.5 wt % of lissamine rhodamine B 1,2 dihexadecanoyl-*sn*-glycero-3-phosphaditylethanolamine (rhodamine-DHPE) [48].

Figure 3.16 Schematic representation of FRAP experiments conducted on an SLB platform. (A) Uniform distribution of fluorescently labelled phospholipid molecules in the SLB, (B) Photobleaching of labeled phospholipid molecules in a particular region of the SLB, (C) Stop bleaching and labeled phospholipids begin to diffuse into the bleached spot, and (D) Recovery of fluorescence intensity in the bleached region and uniform distribution of labeled phospholipids across the SLB.

Figure 3.17 Representative time-lapsed FRAP micrograph snapshots depicting fluorescence recovery of a DOPC SLB. Before FRAP experiment, the SLB was treated with 250 μ M monocaprin treatment. (A) SLB before photobleaching. (B) Time of photobleaching. (C) Recovery of fluorescence intensity in bleached over time.

Figure 3.18 Schematic presentation of MIC assay format using microtiter plates and layout of experimental designs.

Figure 4.1 Chemical structure of tested antimicrobial lipids. Lauric acid, glycerol monolaurate, and SDS each have a 12-carbon long primary alkyl chain. The critical micelle concentrations of the surfactants in aqueous solution are: lauric acid (1.5 mM, Ref. [25]); glycerol monolaurate (42 μ M, Ref. [26]); and SDS (260 μ M, Ref. [26]). The quoted CMC values were obtained from literature reports which investigated the

surfactants in high ionic strength aqueous conditions comparable to the conditions used in this study.

Figure 4.2 QCM-D Investigation of SDS treatment on supported lipid bilayers. Δf (blue line with squares) and ΔD (red line with triangles) shifts as functions of time are presented for (A) 2 mM, (B) 1 mM, (C) 500 μ M, (D) 250 μ M, (E) 125 μ M, and (F) 63 μ M SDS. The critical micelle concentration of SDS is 260 μ M [26]. The initial measurement values correspond to a supported lipid bilayer on the silicon dioxide surface. SDS was added at $t = 5$ min (arrow 1) and a washing step was performed (arrow 2) after the measurement signals stabilized.

Figure 4.3 QCM-D Investigation of lauric acid treatment on supported lipid bilayers. Δf (blue line with squares) and ΔD (red line with triangles) shifts as functions of time are presented for (A) 2 mM, (B) 1 mM, (C) 500 μ M, and (D) 250 μ M lauric acid. The critical micelle concentration of lauric acid is 1.5 mM [25]. The initial measurement values correspond to a supported lipid bilayer on the silicon dioxide surface. Lauric acid was added at $t = 5$ min (arrow 1) and a washing step was performed (arrow 2) after the measurement signals stabilized.

Figure 4.4 QCM-D Investigation of GML treatment on supported lipid bilayers. Δf (blue line with squares) and ΔD (red line with triangles) shifts as functions of time are presented for (A) 2 mM, (B) 1 mM, (C) 500 μ M, (D) 250 μ M, (E) 125 μ M, (F) 63 μ M, (G) 31 μ M, (H) 16 μ M, and (I) 8 μ M GML. The critical micelle concentration of GML is 42 μ M [26]. The initial measurement values correspond to a supported lipid bilayer on the silicon dioxide surface. GML was added at $t = 5$ min (arrow 1) and a washing step was performed (arrow 2) after the measurement signals stabilized.

Figure 4.5 Microscopic observation of SDS-induced solubilization of supported lipid bilayers. (A-I) Image snapshots at various time points during SDS introduction depict nucleation sites that promote tubule formation followed by complete solubilization of the

lipid bilayer. $t = 0$ sec corresponds to the introduction of 2 mM SDS solution into the measurement chamber. The scale bars are 20 μm .

Figure 4.6 Microscopic observation of lauric acid-induced tubule formation on supported lipid bilayers. (A-F) Image snapshots at various time points depict nucleation sites from which stable tubules grow. $t = 0$ sec corresponds to the introduction of 2 mM lauric acid solution into the measurement chamber. (G) Image snapshot after the flow was stopped. (H-I) Image snapshots show the effects of buffer rinsing, including tubule lysis and appearance of fluorophore-poor domains. The scale bars are 20 μm .

Figure 4.7 Microscopic observation of GML-induced spherical protrusions on supported lipid bilayers. (A-F) Image snapshots at various time points depict nucleation sites from which entangled tubules grow and form spherical caps. $t = 0$ sec corresponds to the introduction of 500 μM GML solution into the measurement chamber. (G) Image snapshot after the flow was stopped. (H-I) Image snapshots show the effects of buffer rinsing, including cap lysis as well as the appearance and disappearance of fluorophore-poor regions. The scale bars are 20 μm .

Figure 5.1 Determination of critical micelle concentration using the 1-pyrenecarboxaldehyde fluorescence probe. Peak wavelength is presented as a function of compound concentration in PBS solution for (A) capric acid and (B) monocaprin. The corresponding chemical structures of each compound are presented above each graph. Each data point is the average of six technical replicates ($n = 6$). The average and standard deviation (expressed as the error bars) for each data point are presented where applicable. The CMC value is defined as the highest test concentration at which no peak shift occurs.

Figure 5.2 QCM-D measurement of BSA protein adsorption onto bare and SLB-coated silicon dioxide surfaces. Changes in the QCM-D frequency signal were monitored as a function of time. The measurement baseline was recorded in PBS solution, and BSA protein was added starting at $t=5$ min (see arrow).

Figure 5.3 QCM-D investigation of capric acid treatment on supported lipid bilayers. Δf (blue line with squares) and ΔD (red line with triangles) shifts as a function of time are presented for (A) 8 mM, (B) 4 mM, (C) 2 mM, and (D) 1 mM capric acid. The initial measurement values correspond to a supported lipid bilayer on the silicon dioxide surface. Capric acid was added at $t = 5$ min (arrow 1), and a washing step was performed (arrow 2) after the measurement signals stabilized.

Figure 5.4 QCM-D investigation of monocaprin treatment on supported lipid bilayers. Δf (blue line with squares) and ΔD (red line with triangles) shifts as a function of time are presented for (A) 4 mM, (B) 2 mM, (C) 1 mM, (D) 500 μ M, (E) 250 μ M, (F) 125 μ M, (G) 63 μ M, (H) 31 μ M, and (I) 16 μ M monocaprin. The initial measurement values correspond to a supported lipid bilayer on the silicon dioxide surface. Monocaprin was added at $t = 5$ min (arrow 1), and a washing step was performed (arrow 2) after the measurement signals stabilized.

Figure 5.5 Microscopic observation of 4 mM capric acid-induced tubule formation on supported lipid bilayers. (A–F) Image snapshots at various time points depict nucleation sites from which tubules grow. $t = 0$ min corresponds to the introduction of 4 mM capric acid solution into the measurement chamber. The scale bar is 20 μ m.

Figure 5.6 Microscopic observation of 1 mM capric acid addition to supported lipid bilayers. (A–F) Image snapshots at various time points during after capric acid was added to the supported lipid bilayer. The growth of a small number of tubules was observed. $t = 0$ min corresponds to the introduction of 1 mM capric acid solution into the measurement chamber. The scale bar is 20 μ m.

Figure 5.7 Microscopic observation of 1 mM monocaprin-induced bud protrusions on supported lipid bilayers. (A–F) Image snapshots at various time points depict nucleation sites from which entangled tubules grow and form buds. $t = 0$ min corresponds to the introduction of 1 mM monocaprin solution into the measurement chamber. The scale bar is 20 μ m.

Figure 5.8 Microscopic observation of 250 μM monocaprin-induced tubule formation on supported lipid bilayers. (A–F) Image snapshots at various time points depict nucleation sites from which tubules grow. $t = 0$ min corresponds to the introduction of 250 μM monocaprin solution into the measurement chamber. The scale bar is 20 μm .

Figure 6.1 QCM-D evaluation of cholesterol-rich SLB formation. QCM-D measurements were performed to characterize the SLB formation process. The final (A) Δf shifts and (B) ΔD shifts are reported after SLB formation was completed. The measurement shifts are relative to baseline recordings in equivalent aqueous buffer solution. The molar percentage (mol%) of cholesterol refers to the DOPC phospholipid/cholesterol mixture that was used in the SALB protocol. (C) QCM-D Δf shifts are reported as a function of time for cholesterol extraction from cholesterol-rich SLBs upon M β CD treatment. After SLB formation, the Δf shifts were normalized to baseline values at $t = 0$ min, and 1 mM M β CD was then added starting at $t = 5$ min. Positive Δf shifts indicate cholesterol extraction from the SLB platform. (D) Calculated molar percentage of cholesterol in SLB platform as a function of the input cholesterol fraction in the lipid mixture used in the SALB protocol. Mean and standard deviation (error bars) are reported from five or more technical replicates.

Figure 6.2 Effect of SDS treatment on mass and viscoelastic properties of cholesterol-rich SLBs. QCM-D measurements were performed, and representative Δf (blue squares) and ΔD (red triangles) shifts are reported as a function of time for DOPC phospholipid/cholesterol SLBs with (A) 0, (B) 17, (C) 35, and (D) 52 mol% cholesterol. The initial measurement values correspond to fabricated SLB platforms on the sensor surface. 1 mM SDS was added starting at $t = 5$ min (arrow 1), and a buffer washing step was then performed after the measurement signals stabilized (arrow 2).

Figure 6.3 Effect of LA treatment on mass and viscoelastic properties of cholesterol-rich SLBs. QCM-D measurements were performed, and representative Δf (blue squares) and ΔD (red triangles) shifts are reported as a function of time for DOPC phospholipid/cholesterol SLBs with (A) 0, (B) 17, (C) 35, and (D) 52 mol% cholesterol.

The initial measurement values correspond to fabricated SLB platforms on the sensor surface. 2 mM LA was added starting at $t = 5$ min (arrow 1), and a buffer washing step was then performed after the measured signals stabilized (arrow 2).

Figure 6.4 Effect of GML treatment on mass and viscoelastic properties of cholesterol-rich SLBs. QCM-D measurements were performed, and representative Δf (blue squares) and ΔD (red triangles) shifts are reported as a function of time for DOPC phospholipid/cholesterol SLBs with (A) 0, (B) 17, (C) 35, and (D) 52 mol% cholesterol. The initial measurement values correspond to fabricated SLB platforms on the sensor surface. 125 μ M GML was added starting at $t = 5$ min (arrow 1), and a buffer washing step was then performed after the measurement signals stabilized (arrow 2).

Figure 6.5 Trend in QCM-D measurement shifts for membrane remodeling behavior induced by different test compounds. Maximum Δf and ΔD shifts upon SDS, LA, or GML treatment are presented as a function of cholesterol fraction in the DOPC phospholipid/cholesterol SLB platforms. The net Δf and ΔD shifts are reported as $\Delta f_{\text{measured}} - \Delta f_{\text{bilayer}}$ and $\Delta D_{\text{measured}} - \Delta D_{\text{bilayer}}$ shifts, respectively, in order to normalize the magnitude of the measurement shifts across the different SLB platforms. Mean and standard deviation (error bars) are reported from three or more technical replicates.

Figure 7.1 (A) Chemical structure of glycerol monolaurate (GML) and dodecylglycerol (DDG) and (B) Determination of critical micelle concentration of DDG in PBS [1X, pH 7.2]. Each data point presented in the graph is the average value of six technical replicates ($n = 6$). Standard deviations (expressed as the error bars) are presented where applicable. The CMC value is defined as the highest test concentration before the first peak shift occurs.

Figure 7.2 Microscopic observation of 31 μ M GML- and DDG-induced membrane morphological responses on supported lipid bilayers. Image snapshots at various time points during after addition of (A) 31 μ M GML and (B) 31 μ M DDG to SLB platform are

presented. $t = 0$ min corresponds to the introduction of antimicrobial lipid into the measurement chamber. The scale bars are 20 μm .

Figure 7.3 Determination of critical micelle concentration for GML/LA mixture. Peak wavelength is presented as a function of compound concentration in PBS solution for Different molar fraction of GML/LA mixture (GML:LA) as (A) 10:0, (B) 8:2, (C) 6:4, (D) 4:6, (E) 6:4, and (F) 0:10. Each data point presented in the graph is the average value of six technical replicates ($n = 6$). Standard deviations (expressed as the error bars) are presented where applicable. The CMC value is defined as the highest test concentration before the first peak shift occurs.

Figure 7.4 QCM-D investigation of GML/LA mixture treatment on supported lipid bilayers. Δf (blue line with circle) and ΔD (red line with triangles) shifts are presented as a function of time. Different molar fraction of GML/LA mixture (GML:LA) as (A) 10:0, (B) 8:2, (C) 6:4, (D) 4:6, (E) 6:4, and (F) 0:10 were treated above the corresponding CMC values. The baseline values at $t = 0$ min correspond to an SLB platform on the sensor surface. GML/LA mixture solution was added at $t = 5$ min (arrow 1), and a buffer washing step was performed (arrow 2) after the measurement signals stabilized.

Abbreviations

ABSSSI	Acute Bacterial Skin and Skin Structure Infection
ATP	Adenosine Triphosphate
ATR-FTIR	Attenuated Total Reflection-Fourier Transform Infrared
BSA	Bovine Serum Albumin
C ₁₂ E ₈	Octaethyleneglycol Dodecylether
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CMC	Critical Micelle Concentration
Cryo-TEM	Cryogenic Transmission Electron Microscopy
DDG	1-O-dodecyl-rac-glycerol
DHA	Docosahexaenoic Acid
DMPC	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphocholin
DMSO	Dimethyl Sulfoxide
DMTAP	1,2-dimyristoyl- <i>sn</i> -glycero-3-trimethylammoniumpropan
DOPA	Dioleoylphosphatidic Acid
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FRAP	Fluorescence Recovery After Photobleaching
GML	Glycerol Monolaurate
GRAS	Generally Recognized As Safe
GTase	Glucosyltransferase
GUV	Giant Unilamellar Vesicle
LA	Lauric Acid
LB	Luria-Bertani
LPC	Lysophosphatidylcholine
LUV	Large Unilamellar Vesicle
MBC	Minimum Bactericidal Concentration
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration

MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin-susceptible <i>Staphylococcus aureus</i>
M β CD	Methyl- β -cyclodextrin
PB	Phosphate Buffer
PBS	Phosphate-buffered Saline
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
QCM-D	Quartz Crystal Microbalance with Dissipation
Rhodamine-DHPE	Lissamine Rhodamine B 1,2-dihexadecanoyl- <i>sn</i> -glycero-3-phosphatidylethanolamine
SALB	Solvent Assisted Lipid Bilayer
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SLB	Supported Lipid Bilayer
SUV	Small Unilamellar Vesicle
Tail-labeled NBD-PC	Tail-labeled Nitrobenzofuran-conjugated PC
TEM	Transmission Electron Microscopy
TRITC	Tetramethylrhodamine

Chapter 1

Introduction

Antimicrobial lipids – single-chain lipid amphiphiles that disrupt bacterial cell membranes – are promising candidates for next-generation antibacterial agents to address the public health needs raised by the growing prevalence of antibiotic-resistant bacteria. To realize this opportunity and other biotechnology applications, a detailed understanding of how antimicrobial lipids interact with and destabilize phospholipid membranes is needed. One of the most important priorities is to unravel the physicochemical basis governing how molecular properties of antimicrobial lipids dictate the scope and potency of biological activities. To achieve this goal, materials science strategies combined with biophysical measurement tools offer outstanding advantages and present new approaches for studying antimicrobial lipids. This chapter presents a brief background of the research field and outlines the problem statement motivating this research topic along with specific objectives and hypotheses to be addressed in this thesis.

1.1 Hypothesis/Problem Statement

One of the greatest public health problems in the world today is the growing rise of antibiotic-resistant bacteria and the associated challenges to treat and prevent bacterial infections [1]. Less than a century ago, the world's first antibiotic, penicillin, was discovered, and the specificity of antibiotics to inhibit bacterial enzymes and other proteins necessary for bacterial cell function proved highly effective, as evidenced by remarkable improvements in healthcare capabilities to treat bacterial infections. As a result, many formerly fatal or debilitating diseases caused by bacterial pathogens suddenly became curable with antibiotic treatment [2].

With high potency and working against a broad spectrum of bacterial targets, antibiotics became the standard drug option to treat bacterial infections, and are also widely used as precautionary measures to treat suspected infections, even when the microbial origin is unknown and could be either bacterial, fungal, or viral among other possibilities. Antibiotics are also administered prophylactically in cases where bacterial infections might arise, such as after surgical operations. In addition, antibiotics are commonly used in the agricultural sector to not only treat and prevent bacterial infections among livestock, but also serve as growth promoters to accelerate the time to reach maturity as well as increase the body mass of animals. For all these reasons, antibiotics have become ubiquitous in society and have had an outsized role in shaping modern life.

However, despite numerous benefits, the drawbacks of antibiotics being so widely prevalent are now becoming apparent as well. With increasing exposure to antibiotics and corresponding selective pressure, bacteria have evolved to become resistant to many antibiotics, and antibiotic-resistant bacteria are widespread. As a result, existing antibiotics are losing their effectiveness to treat bacterial infections, and the problem is further compounded by the dearth of new antibiotics that have been discovered in recent years. In part, the problem is economic because pharmaceutical companies have had weak interest in developing new antibiotics due to low price points, however, the more pressing scientific issue is that the chemical space available for identifying and refining antibiotics is limited. There is growing recognition that society faces an impending post-antibiotic era [3], and hence there is an urgent need to develop new classes of

antibacterial agents.

To address this problem, antimicrobial lipids – single-chain lipid amphiphiles that destabilize microbial membranes – are attractive candidates to become next-generation antibacterial agents for treating bacterial infections. Curiously, the antibacterial properties of antimicrobial lipids have been known since early reports by Dr. Robert Koch and colleagues in the late 1880's, when it was shown that fatty acids, a prominent class of antimicrobial lipid, inhibited growth of the *Bacillus anthracis* pathogen that causes anthrax [4]. Despite strong promise and demonstrated results, antimicrobial lipids faded away by the 1940's due to the emergence of antibiotics, but have received renewed attention amidst the growing impact of antibiotic-resistant bacteria. Indeed, one attractive feature of antimicrobial lipids is that it is difficult for bacteria to mutate to become resistant to them. As such, bacterial cell cultures can be grown in the presence of antimicrobial lipids (at sub-lethal concentrations) for at least one year, without signs of drug-resistant strains emerging [5].

In this thesis, attention is drawn to two particular classes of antimicrobial lipid, namely fatty acids (hydrocarbon chains with a carboxylic acid functional group [6]) and monoglycerides (esterified adducts of a fatty acid and glycerol molecule). The motivation for studying these two classes of antimicrobial lipid arises from pioneering studies by Kabara and colleagues in the 1970's, which systematically investigated the antibacterial potency of medium-chain saturated fatty acids and monoglycerides with different chain lengths [7-10]. It was discovered that lauric acid (LA), which possesses a 12 carbon-long chain, had the most potent activity to inhibit growth of Gram-positive bacteria, and its monoglyceride derivative, glycerol monolaurate (GML), exhibited even stronger inhibitory activity than lauric acid. Importantly, both LA and GML are Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration [11] and abundant in nature. These factors have led to wide exploration of LA and GML for anti-infective applications [12-14], including application topics such as agriculture [15] and cosmetics [6, 16, 17]. Other antimicrobial lipids such as capric acid, which possesses a 10 carbon-long chain, and its monoglyceride derivative, monocaprin, have also received attention, although clarifying how the physicochemical properties of antimicrobial lipids influence biological activities remains an outstanding goal in many respects.

To date, the primary means of assessing the activity profile of an antimicrobial lipid has been to evaluate how treatment affects bacterial cell growth, with the minimum inhibitory concentration (MIC) of test compound being defined as the drug concentration at which no visible growth of bacteria occurs. While such information provides insight into the scope and potency of an antimicrobial lipid, it does not reveal mechanistic information and there is growing interest to understand how antimicrobial lipids destabilize bacterial cell membranes. Biological assays have identified that antimicrobial lipids act as bacteriostatic (growth-inhibiting) or bactericidal (killing) agents depending on the drug concentration, target bacterium and other environmental factors [6, 18]. To directly observe morphological effects on bacterial cell membranes, electron microscopy techniques have been utilized to image bacterial specimens after treatment with antimicrobial lipids [19-25]. While this approach provides visualization of membrane damage, very high concentrations of antimicrobial lipid are typically used (5-10 mM) and the bacterial cells can only be examined after treatment and sample fixation. Similar issues exist with atomic force microscopy experiments for examining the morphology of treated bacterial cells. In general, it is difficult to resolve molecular-level interaction kinetics when working with complex biological samples such as whole bacterial cells, thereby motivating the development of model systems.

To obtain insights into real-time interaction kinetics with a more focused approach, a variety of solution-phase model membrane platforms based on small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) have been employed in combination with measurement techniques such as dynamic light scattering in order to detect how antimicrobial lipids cause membrane destabilization *via* partial solubilization as well as membrane fission [26-33]. These model systems allow detailed characterization of membrane morphological changes by using well-controlled, simplified phospholipid compositions that mimic more complex biological membranes. Time-lapsed optical microscopy imaging of giant unilamellar vesicles (GUVs) has also enabled direct visualization of membrane morphological responses, including swelling, fusion and fission behaviors [34, 35]. Within this scope, supported lipid bilayers (SLBs) are two-dimensional phospholipid bilayers that have emerged as a particularly useful model membrane platform because they can be studied with a wide range of surface-sensitive

measurement techniques, revealing insights into the mass, viscoelastic, fluidic and morphological properties of SLB platforms. Indeed, one particular advantage of SLB platforms is that the two-dimensional phospholipid bilayer can remodel in response to an applied membrane strain, giving rise to three-dimensional morphological responses. However, to date, there has been scant attention to employ SLB platforms for investigating antimicrobial lipids, with only one study reporting the interactions between a long-chain fatty acid and single-component, zwitterionic phospholipid SLB platform and another study investigating the interaction between a short-chain monoglyceride and SLBs derived from bacterial cell membrane extracts [36-38]. Extending such approaches to investigate medium-chain saturated fatty acids and monoglycerides – representing the subset of antimicrobial lipids with the highest activity – is warranted in order to develop model systems for profiling the scope and potency of these antimicrobial lipids. More importantly, moving beyond particular experimental approaches, there is an outstanding need to establish a comprehensive framework for correlating the physicochemical properties of antimicrobial lipids with their corresponding biophysical and biological activities. Key questions that remain unanswered include:

- Do medium-chain saturated fatty acids and monoglycerides interact with phospholipid membranes in distinct ways or follow similar patterns, and what kinds of membrane morphological changes occur?
- How do the physicochemical properties of fatty acids and monoglycerides such as net charge and chain length affect the resulting interactions with supported lipid bilayer platforms?
- What factors determine the minimum bulk concentration at which an antimicrobial lipid destabilizes phospholipid membranes, and how do such behaviors correlate with antibacterial activity?
- How do antimicrobial lipids interact with more complex, biologically relevant membranes in different phase states as compared to simplified, fluid-phase models? For example, how does the presence of sterols in phospholipid-sterol membranes influence interaction kinetics?

To address these outstanding questions and more, the goal of this thesis is to develop SLB platforms for investigating how medium-chain saturated fatty acids and monoglycerides interact with phospholipid membranes, and to correlate concentration-dependent interaction profiles with corresponding molecular self-assembly properties and antibacterial activities. Towards this goal, the overall hypothesis of this thesis is that supported lipid bilayers can provide a predictive model system to evaluate the mechanism of action and potency of antimicrobial lipids, within the broader context of establishing an experimental framework to correlate the molecular self-assembly of antimicrobial lipids with their corresponding biophysical and biological activities. The following specific hypotheses are addressed in this thesis:

- The interactions of medium-chain saturated fatty acids and monoglycerides with supported lipid bilayers induce distinct membrane morphological responses that relate to their different physicochemical properties.
- The molecular self-assembly of fatty acids and monoglycerides is a critical determinant of how these molecules interact with supported lipid bilayers.
- The concentration-dependent activity profiles of fatty acids and monoglycerides interacting with supported lipid bilayers are correlated with the antibacterial potency of these compounds.
- The presence, and fraction thereof, of sterols in supported lipid bilayers modulates the extent to which fatty acids and monoglycerides can induce membrane morphological responses.

1.2 Objectives and Scope

Antimicrobial lipids such as fatty acids and monoglycerides are promising antibacterial agents that destabilize bacterial cell membranes, representing a new approach that might overcome the challenges of antibiotic-resistant bacteria while targeting a bacterial component that has a high barrier to the emergence of resistant strains. At present, it is known that antimicrobial lipids act against bacterial cell membranes, however, it remains to be understood how the physicochemical properties of

antimicrobial lipids affect the corresponding interactions with bacterial cell membranes, including the types of interactions and potency. For example, under physiological pH conditions, fatty acid molecules are anionic while monoglycerides are nonionic. Such differences suggest that the mechanisms by which these compounds interact with phospholipid membranes will show variations as well. To address these outstanding questions, supported lipid bilayers mimicking bacterial cell membranes are promising experimental platforms, especially when combined with appropriate surface-sensitive measurement techniques and complementary measurement approaches to determine the molecular self-assembly of antimicrobial lipids in bulk solution. The measurement responses can be further compared to antibacterial evaluations of antimicrobial lipids that are conducted in equivalent solution conditions and concentration ranges, and hence correlations across biophysical and biological measurements can be established.

Within this scope, the main objective of this work was to develop a comprehensive physicochemical understanding of how fatty acids and monoglycerides interact with phospholipid membranes, and supported lipid bilayers were selected as the model experimental system. While other model membrane platforms such as GUVs have been utilized to study interactions between antimicrobial lipids and phospholipid membranes to a limited extent, it has been difficult to resolve molecular-level interaction kinetics and such platforms require specialized solution conditions (*e.g.*, high concentrations of glucose and/or sucrose), which can affect the molecular self-assembly properties of the antimicrobial lipid molecules and corresponding interactions with phospholipid membranes. In contrast, SLB platforms possess greater versatility and can be studied with a wide range of surface-sensitive measurement techniques based on different physical principles. The compelling features of SLB platforms are further enhanced by the broader fabrication opportunities that are afforded by the recently developed solvent-assisted lipid bilayer (SALB) method, and the scope of fabrication possibilities now extends to preparing SLBs from mixtures of phospholipids and sterols with controllable molar ratio [39-41]. To accomplish this objective, the following studies were conducted in order to establish the measurement platform and analytical methodologies as well as to investigate selected antimicrobial lipids comprised of highly active, medium-chain saturated fatty acids and monoglycerides:

- An SLB platform was established to investigate the membrane morphological responses induced by representative fatty acids and monoglycerides, namely LA and GML. These two compounds were chosen because, among medium-chain antimicrobial lipids, they have the highest reported antibacterial activity against Gram-positive bacteria. The concentration-dependent activity of the two compounds acting against SLBs was first investigated by quartz crystal microbalance-dissipation (QCM-D) experiments, revealing unique patterns of membrane morphological response based on measurement signatures that are sensitive to the mass and viscoelastic properties of the SLB platform. Treatment of fluorescently labeled SLB platforms with selected active concentrations of LA and GML was also conducted, while fluorescence microscopy experiments were performed to directly observe membrane morphological changes and clarify the origins of the distinct QCM-D measurement responses. This integrated approach facilitated the development of an experimental framework to characterize and classify the interactions of antimicrobial lipids with SLB platforms. Additional microbiological tests were conducted to determine the MIC values of LA and GML for inhibiting growth of two *S. aureus* strains, and to correlate concentration-dependent membrane morphological responses with this biological activity. This work set the pretext for understanding how the physicochemical properties of fatty acids and monoglycerides influence interactions with phospholipid membranes, and for establishing broadly applicable experimental framework to study antimicrobial lipids in general.
- Building on this work, the measurement capabilities were next extended to study capric acid and monocaprin, with a particular focus on clarifying the role of molecular self-assembly in dictating the biophysical activity of antimicrobial lipids. Capric acid and monocaprin were selected because they are antimicrobial lipids with 10-carbon long chains that also have high antibacterial activity, especially against bacteria commonly associated with foodborne illnesses [42]. A fluorescence spectroscopy method based on measuring the extinction spectra of a fluorescent, small molecule probe that is sensitive to the surrounding dielectric environment, was established to measure the CMC values of capric acid and

monocaprin, and design experiments to evaluate the activity profile of the test compounds above and below the corresponding CMC values. The concentration-dependent activities of the two compounds acting against SLB platforms were next investigated by QCM-D and fluorescence microscopy experiments, and the range of active concentrations was compared to the corresponding CMC values. Fluorescence recovery after photobleaching (FRAP) experiments were also conducted in order to determine how compound treatment affects membrane fluidity. Together, the results provided guidance into how the molecular self-assembly of fatty acids and monoglycerides influences corresponding interactions with SLB platforms, not only in terms of morphological properties but also membrane fluidity.

- While the foregoing work focused on investigating how antimicrobial lipids affect the morphological properties of SLB platforms composed of single-component, zwitterionic phospholipids, extending such approaches to SLB platforms composed of both phospholipids and sterols is warranted to better understand how antimicrobial lipids act against biologically relevant membrane compositions. To achieve this goal, SLB platforms enriched in varying fractions of cholesterol were fabricated by the solvent-assisted lipid bilayer (SALB) method. QCM-D measurements were conducted to characterize the mass and viscoelastic properties of the SLB platforms and the molar fraction of cholesterol was further quantified; the combination of measurement signatures enabled identification of the phase state(s) of the phospholipid-cholesterol SLB platforms. The effects of cholesterol fraction on membrane morphological responses induced by active concentrations of LA and GML were further investigated by QCM-D measurements, and provided the basis for clarifying how sterols influence the viscoelastic properties of phospholipid membranes and in turn act as mediators of membrane stress relaxation.

1.3 Dissertation Overview

This thesis is organized into the following chapters:

Chapter 1: “Introduction” presents the background of the research field and outlines the scientific rationale motivating the Ph.D. research topic, including elaboration of key goals and project scope.

Chapter 2: “Literature Review” introduces a general background of antimicrobial lipids, including classification scheme, and also provides a comprehensive review of the antibacterial spectrum and potency of molecules within this class along with existing knowledge about mechanistic underpinnings. Characterization methods to evaluate the biological and biophysical activities of antimicrobial lipids are introduced, including critical discussion of the type of information that is obtained with different methods. In closing, outstanding questions are highlighted that motivate the research goals of this thesis.

Chapter 3: “Experimental Methodology” provides a description of materials, materials synthesis, and experimental techniques along with detailed explanation of the physical principles behind the different techniques.

Chapter 4: “Spectrum of Membrane Morphological Responses to Antibacterial Fatty Acids and Related Surfactants” describes the first empirical observations reporting how fatty acids and monoglycerides, namely LA and GML, induce distinct membrane morphological responses in SLB platforms. Furthermore, the chapter establishes how the physicochemical properties of different classes of antimicrobial lipid influence the scope and potency of biophysical and biological activities, and provides a physical explanation to relate membrane morphological responses to applied membrane strain.

Chapter 5: “Correlating Membrane Morphological Responses with Micellar Aggregation Behavior of Capric Acid and Monocaprin” presents fundamental characterization relating the molecular self-assembly of fatty acids and monoglycerides to different types of membrane morphological responses in SLB platforms. The influence of monomeric *vs.* micellar states on membrane morphological responses is identified for each class of antimicrobial lipid, and further extended to understanding how antimicrobial lipids

influence the fluidic properties of SLB platforms as well.

Chapter 6: “Understanding How Sterols Regulate Membrane Remodeling in Supported Lipid Bilayers” discusses how the presence of sterols in SLB platforms influences membrane morphological responses induced by antimicrobial lipids. The chapter also introduces improved methods for fabricating cholesterol-enriched SLB platforms and presents detailed characterization of how cholesterol fraction influences the membrane phase state(s) in the SLB platform.

Chapter 7: “Discussion and Future Work” summarizes key findings of the work while drawing out particularly significant findings that advance the field and discussing future research opportunities. Particular attention is placed on understanding how antimicrobial lipids and engineered variants thereof function in biologically relevant environments as well as on how mixtures of different antimicrobial might act synergistically to yield unique patterns of membrane morphological responses.

1.4 Findings and Outcomes/Originality

The findings achieved in this work led to several novel outcomes that advance measurement capabilities to investigate the activity profile of antimicrobial lipids and to classify the potency of and mechanisms by which different classes of antimicrobial lipid operate. Within this scope, the key findings of this thesis can be summarized as follows:

1. SLB platforms were established to characterize the mechanism of action of antimicrobial lipids, namely medium-chain saturated fatty acids and monoglycerides. By employing advanced fabrication strategies, it was possible to form SLBs ranging from single-component, zwitterionic phospholipid compositions to complex phospholipid-cholesterol mixtures in different phase states. Rigorous characterization of SLB fabrication ensured high-quality platforms for detecting the activity of antimicrobial lipids, and these efforts represent the first attempt to systematically investigate how SLB platform composition influences the activity of antimicrobial lipids. Importantly, an integrated experimental framework was utilized to monitor how antimicrobial

lipids trigger membrane morphological responses in the SLB platforms and to correlate such activities with the bulk molecular self-assembly state of the tested compounds.

2. It was discovered that different classes of antimicrobial lipid elicit distinct patterns of membrane morphological responses in SLB platforms. This finding represents a significant advance because there is scant discussion in the scientific literature about mechanistic details underpinning the activity of fatty acids and monoglycerides, and the only reported comparisons, to date, are about biological activities, *e.g.*, level of inhibitory activity against bacterial growth. By utilizing the SLB platform, it was identified that anionic fatty acids induce the formation of elongated tubule structures protruding from the SLB platform while nonionic monoglycerides caused the formation of spherical buds and caps. Building on these empirical observations, the distinct responses were rationalized by taking into account how the net charge of an antimicrobial lipid influences its rate of membrane translocation and corresponding effects on membrane strain profile, including strain symmetry/asymmetry across either one or both bilayer leaflets.
3. While different classes of antimicrobial lipid induce distinct membrane morphological responses, it was determined that the molecular self-assembly state of an antimicrobial lipid is an important governing parameter that influences the activity profile of all tested antimicrobial lipids. Specifically, it was identified that antimicrobial lipids are typically active against SLBs when the bulk concentration of compound is around the corresponding CMC value or higher. Fatty acids were active against SLBs only above the corresponding CMC value in which case they induced tubule formation, whereas no activity was detected below the CMC value. On the other hand, when the bulk concentration of monoglycerides was above the corresponding CMC value, they induced membrane budding, while slightly lower concentrations below the CMC value induced tubule formation in some cases. At appreciably lower concentrations, monoglycerides were inactive against SLBs. Hence, both fatty acids and monoglycerides are strongly influenced by self-assembled molecular state (monomer *vs.* micelle) and particular differences in concentration-dependent

activity profiles were further explained by variations in molecular properties, including net charge, chain length, and hydrogen-bonding capacity.

4. The concentration-dependent pattern by which antimicrobial lipids act against SLB platforms was found to correlate with minimum inhibitory concentrations (MICs) of test compound that prevented visible growth of a model Gram-positive bacterium, *Staphylococcus aureus*. In particular, the CMC value of an antimicrobial lipid was closely related to its corresponding MIC value, supporting that antibacterial activities are linked to membrane-destabilizing activities. In a broader context, this finding also helps to explain why monoglycerides are more potent antibacterial agents than their fatty acid equivalents as it is thermodynamically favorable for nonionic monoglycerides to form micellar aggregates at lower bulk concentrations than anionic fatty acids. In addition, the observed correlation between biophysical and biological activities validates the utility of employing SLB platforms to characterize the scope and potency of antimicrobial lipids and generate information about structure-activity relationships before proceeding to biological studies.
5. The presence of cholesterol in the SLB platform influenced the extent of membrane morphological responses, in line with the biological role of sterols as mediators of membrane stress relaxation. With increasing cholesterol fraction in the SLB platform, fatty acid addition caused larger measurement responses associated with tubule formation. In contrast, monoglyceride addition led to smaller measurement responses associated with membrane budding. The different effects of cholesterol on promoting or inhibiting membrane morphological responses, which depended on the identity of the perturbing antimicrobial lipid, were explained by how cholesterol mediates stress relaxation, the corresponding influence of cholesterol on the elastic (stiffness) and viscous (stress relaxation) properties of SLB platforms, and the membrane translocation properties of fatty acids and monoglycerides. Altogether, these findings reinforced that antimicrobial lipids interact with biologically relevant, complex membranes in a similar manner to how they interact with simplified version, and provided a physicochemical explanation to understand how sterols influence the

extent of membrane remodeling as part of membrane morphological responses.

References

- [1] R. Laxminarayan, A. Duse, C. Wattal, A.K. Zaidi, H.F. Wertheim, N. Sumpradit, E. Vlieghe, G.L. Hara, I.M. Gould, H. Goossens, *The Lancet Infectious Diseases*, 13 (2013) 1057-1098.
- [2] R.I. Aminov, *Frontiers in Microbiology*, 1 (2010).
- [3] A.J. Alanis, *Archives of Medical Research*, 36 (2005) 697-705.
- [4] H. Thormar, *Lipids and Essential Oils as Antimicrobial Agents*, John Wiley & Sons, (2010).
- [5] J. Davies, D. Davies, *Microbiology and Molecular Biology Reviews*, 74 (2010) 417-433.
- [6] A.P. Desbois, V.J. Smith, *Applied Microbiology and Biotechnology*, 85 (2010) 1629-1642.
- [7] J.J. Kabara, D.M. Swieczkowski, A.J. Conley, J.P. Truant, *Antimicrobial Agents and Chemotherapy*, 2 (1972) 23-28.
- [8] J. Kabara, R. Vrable, M.L.K. Jie, *Lipids*, 12 (1977) 753-759.
- [9] J.J. Kabara, *Journal of the American Oil Chemists' Society*, 61 (1984) 397-403.
- [10] J.J. Kabara, *Journal of the Society of Cosmetic Chemists*, 29 (1978) 733-741.
- [11] J.J. Kabara, *Journal of the Society of Cosmetic Chemists*, 31 (1980) 1-10.
- [12] T. Kitahara, N. Koyama, J. Matsuda, Y. Aoyama, Y. Hirakata, S. Kamihira, S. Kohno, M. Nakashima, H. Sasaki, *Biological and Pharmaceutical Bulletin*, 27 (2004) 1321-1326.
- [13] T. Nakatsuji, M.C. Kao, J.-Y. Fang, C.C. Zouboulis, L. Zhang, R.L. Gallo, C.-M. Huang, *Journal of Investigative Dermatology*, 129 (2009) 2480-2488.
- [14] P.M. Schlievert, M.L. Peterson, *PloS one*, 7 (2012) e40350.
- [15] E. Skřivanová, Z. Molatová, M. Marounek, *Veterinary Microbiology*, 126 (2008) 372-376.
- [16] A. P Desbois, *Recent Patents on Anti-Infective Drug Discovery*, 7 (2012) 111-122.
- [17] H. Maag, *Journal of the American Oil Chemists' Society*, 61 (1984) 259-267.
- [18] H. Heerklotz, *Quarterly Reviews of Biophysics*, 41 (2008) 205-264.

- [19] D.P. Speert, L.W. Wannamaker, E.D. Gray, C.C. Clawson, *Infection and Immunity*, 26 (1979) 1202-1210.
- [20] H.R. Knapp, M.A. Melly, *Journal of Infectious Diseases*, 154 (1986) 84-94.
- [21] L.-L. Wang, E.A. Johnson, *Applied and Environmental Microbiology*, 58 (1992) 624-629.
- [22] G. Bergsson, J. Arnfinnsson, S.M. Karlsson, Ó. Steingrímsson, H. Thormar, *Antimicrobial Agents and Chemotherapy*, 42 (1998) 2290-2294.
- [23] G. Bergsson, J. Arnfinnsson, Ó. Steingrímsson, H. Thormar, *Journal of Pathology, Microbiology and Immunology*, 109 (2001) 670-678.
- [24] E. Skřivanová, M. Marounek, G. Dlouha, J. Kaňka, *Letters in Applied Microbiology*, 41 (2005) 77-81.
- [25] S. Shin, V. Bajpai, H. Kim, S. Kang, *LWT-Food Science and Technology*, 40 (2007) 1515-1519.
- [26] S. Lonchin, P.L. Luisi, P. Walde, B.H. Robinson, *The Journal of Physical Chemistry B*, 103 (1999) 10910-10916.
- [27] N. Berclaz, M. Müller, P. Walde, P.L. Luisi, *The Journal of Physical Chemistry B*, 105 (2001) 1056-1064.
- [28] N. Berclaz, E. Blöchliger, M. Müller, P.L. Luisi, *The Journal of Physical Chemistry B*, 105 (2001) 1065-1071.
- [29] S. Rasi, F. Mavelli, P.L. Luisi, *The Journal of Physical Chemistry B*, 107 (2003) 14068-14076.
- [30] S. Chungcharoenwattana, M. Ueno, *Chemical and Pharmaceutical Bulletin*, 52 (2004) 1058-1062.
- [31] S. Chungcharoenwattana, H. Kashiwagi, M. Ueno, *Colloid and Polymer Science*, 283 (2005) 1180-1189.
- [32] S. Chungcharoenwattana, M. Ueno, *Chemical and Pharmaceutical Bulletin*, 53 (2005) 260-262.
- [33] M.L. Rogerson, B.H. Robinson, S. Bucak, P. Walde, *Colloids and Surfaces B: Biointerfaces*, 48 (2006) 24-34.
- [34] P. Peterlin, V. Arrigler, K. Kogej, S. Svetina, P. Walde, *Chemistry and Physics of Lipids*, 159 (2009) 67-76.

- [35] M. Mally, P. Peterlin, S.a. Svetina, *The Journal of Physical Chemistry B*, 117 (2013) 12086-12094.
- [36] D. Thid, J.J. Benkoski, S. Svedhem, B. Kasemo, J. Gold, *Langmuir*, 23 (2007) 5878-5881.
- [37] K.R. Flynn, L.L. Martin, M.L. Ackland, A.A. Torriero, *Langmuir*, 32 (2016) 11717-11727.
- [38] M. Hyldgaard, D.S. Sutherland, M. Sundh, T. Mygind, R.L. Meyer, *Applied and Environmental Microbiology*, 78 (2012) 2957-2965.
- [39] S.R. Tabaei, J.-H. Choi, G. Haw Zan, V.P. Zhdanov, N.-J. Cho, *Langmuir*, 30 (2014) 10363-10373.
- [40] S.R. Tabaei, J.A. Jackman, M. Kim, S. Yorulmaz, S. Vafaei, N.-J. Cho, *Journal of Visualized Experiments: JoVE*, (2015).
- [41] S.R. Tabaei, J.A. Jackman, S.-O. Kim, B. Liedberg, W. Knoll, A.N. Parikh, N.-J. Cho, *Langmuir*, 30 (2014) 13345-13352.
- [42] H. Thormar, H. Hilmarsson, G. Bergsson, *Applied and Environmental Microbiology*, 72 (2006) 522-526.

Chapter 2

Literature Review

Antimicrobial lipids such as fatty acid and monoglycerides are promising antibacterial agents that destabilize bacterial cell membranes. While the antimicrobial scope and potency of antimicrobial lipids is widely studied, the current body of knowledge remains largely empirical based on indirect biological readouts. A fundamental physicochemical understanding to explain how the structure of antimicrobial lipids influences biophysical and biological activities centered around interactions with phospholipid membranes needs to be established. In this chapter, a background of antimicrobial lipids, including classification of fatty acids and molecular derivatives, is provided along with description of their antibacterial spectrum and currently understood mechanisms. Building on this knowledge, detailed discussion of different experimental approaches to evaluate biological and biophysical activities of antimicrobial lipids is provided along with critical remarks motivating the need for future analytical improvements. Finally, the outstanding questions motivating the research goals of this thesis are formulated and focus on connecting biophysical and biological activities of antimicrobial lipids with a broader understanding of how molecular self-assembly and interfacial science drives interactions with phospholipid membranes.

2.1 Antimicrobial Lipids

2.1.1 Historical Origins

The field of antimicrobial lipids originated in the late 19th century when the antibacterial properties of soaps were gaining attention for disinfectant applications. One of the earliest observations took place in 1881 when the esteemed microbiologist Dr. Robert Koch noted that fatty acids – a key component of soaps – can inhibit the growth of *Bacillus anthracis*, which is a medically important bacterium that causes anthrax. Sparked by this scientific discovery and other ones involving an antibacterial enzyme called lysozyme, there were growing calls to utilize antibacterial compounds for the treatment of bacterial infections in the early 20th century. Antimicrobial lipids were a particularly attractive option with potent activity against numerous, medically important bacteria [1, 2]. This promise was further reinforced by later findings in the 1940's by Burtenshaw and colleagues, which showed that antimicrobial lipids are an important component of the human skin's innate immune system [3, 4]. As part of the innate immune system, there is also another class of antimicrobial natural product that is comprised of membrane-active, typically cationic amphipathic peptides (termed "antimicrobial peptides"). Inspired by the innate immune system, there has long been interest in developing therapeutic strategies based on exogenous addition of these natural antimicrobial peptides or synthetic peptides with engineered properties. Such peptides can interact with lipid membranes via a variety of mechanisms involving pore formation and/or membrane solubilization. Notably, the peptides can often display high potency (down to nanomolar concentrations) against bacterial pathogens and exhibit some degree of selectivity by preferentially targeting negatively charged lipid compositions that are more prominent in bacterial cell membranes than the more conventionally zwitterionic ones found in human cell membranes. At the same time, peptides are prone to enzymatic degradation by proteases, have weak performance and limited selectivity in physiological salt conditions, low environmental stability, and high production costs. For these reasons, antimicrobial lipids have received significant attention due to their distinct mechanism of action, abundant and affordable supply, and greater stability for sustained performance.

Indeed, the development of therapeutic strategies of antimicrobial lipids based on exogenous addition of natural compounds was viewed as medically opportune.

However, the impact of antimicrobial lipids was short-lived, not due to their own shortcomings but rather due to the rise of other classes of antibacterial agents. The world's first antibiotic, penicillin, was discovered in the late 1940's and revolutionized the treatment of bacterial infections, leading to marked improvements in human healthcare [5]. While antimicrobial lipids broadly target bacterial cell membranes, antibiotics like penicillin specifically inhibit the function of bacterial enzymes. As a result, antibiotics have very high potency and became the standard treatment for bacterial infections, a fact which remains true today. So successful were antibiotics that they became routinely used for not only therapeutic treatment of diagnosed bacterial infections in humans, but were also utilized for therapeutic treatment of animals as well as prophylactic applications involving both humans and animals. In many cases even today, antibiotics are viewed as precautionary measures to treat suspected infections, even when it is unknown whether the pathogen has bacterial, viral, or fungal origins. In addition, antibiotics are commonly given to animals in the agricultural sector to not only ward off disease, but also accelerate growth and increase body mass. For all these reasons, antibiotics are ubiquitous in modern society.

As time progresses, it has become clear that the prolific use of antibiotics has come at a cost. With increasing exposure to antibiotics, bacteria have evolved to become resistant to antibiotics in many cases, and antibiotic resistance is currently one of the world's leading public health issues [6]. For example, antibiotics that were once routinely given to patients for treating certain infections no longer work against these infections, and such predicaments are having a dramatic impact on healthcare and medicine. The challenges are compounded by the reality that few companies have searched for new antibiotics due to scant economic opportunity – only four new classes of antibiotics have been reported in the past half-century – and the limited number of bacterial targets that are “druggable”. Current drug development efforts are mainly aimed at overcoming resistant strains, either by synthesizing derivatives of existing antibiotics with minor variations in chemical structure or devising delivery strategies to resurrect the inhibit function of existing antibiotics. Even with growing public support to catalyze antibiotic

discovery and development, future growth opportunities are bleak. As a result, there are fears that we are entering a post-antibiotic era where antibiotics become obsolete and new antibacterial agents must be developed.

Within this scope, antimicrobial lipids deserve renewed attention as candidate antibacterial agents. There are numerous practical benefits to explore them. Many antimicrobial lipids are recognized as safe by regulatory agencies [7] and work against a wide range of bacteria, viruses, and fungi [2, 8-10]. Retrospectively, it has also become increasingly apparent that antimicrobial lipids are useful for various applications, including agriculture [11], food, and cosmetics [8, 12, 13]. Scientifically, the proposed mechanisms of action of antimicrobial lipids suggests plausible routes to sustainable use by avoiding the emergence of drug-resistant bacterial strains. As mentioned above, antimicrobial lipids interfere with bacterial cell membranes and can cause membrane lysis and cell death or inhibit bacterial functions and hence prevent growth [8]. Because antimicrobial lipids do not target a specific component that can evolve, there is a much higher barrier to the emergence of drug-resistant bacterial strains. As such, there is growing motivation to characterize antimicrobial lipids and gain deeper insight into their mechanisms of action.

At present, one long-standing challenge of employing antimicrobial lipids has been limited understanding of how their physicochemical properties are linked with biological activities. Conventionally, biological assays are conducted to determine if an antimicrobial lipid kills a bacterium or inhibits its growth. Some work in this direction has helped to establish structure-activity relationships as will be explained below, however, there remains outstanding needs to connect the physical chemistry of antimicrobial lipids with biological activities.

2.1.2 Classification

For the purposes of this thesis, antimicrobial lipids are defined as single-chain lipid amphiphiles that interact with bacterial cell membranes and exhibit antibacterial activity. Fatty acids are a widely studied type of antimicrobial lipid, and are composed of a single saturated or unsaturated hydrocarbon chain and a carboxylic acid group on one end. As

such, fatty acids are amphipathic molecules, with the hydrocarbon chain constituting the hydrophobic part while the carboxylic acid group is hydrophilic (either polar or anionic in aqueous solutions, depending on pH conditions). For example, the carboxylic acid groups of medium-chain fatty such as those reported in this thesis, have pK_a values around pH 5 [14], and therefore the fatty acids are anionic (deprotonated carboxylic acid groups) around the physiological (blood) pH condition of 7.4. In addition to fatty acids, other derivatives have been reported to have antibacterial activity, with one prominent class being monoglycerides that are composed of a fatty acid connected with a glycerol molecule *via* an ester bond. Other synthetic versions are also possible such as related compounds with ether bonds, rendering such molecules impervious to bacterial lipases.

Figure 2.1 Chemical structures of fatty acids and monoglycerides. Saturated fatty acids; Capric acid (C10:0), Lauric acid (C12:0), Monoglycerides; Monocaprin (MG C10:0), Glycerol monolaurate (MG C12:0), Unsaturated fatty acids: Oleic acid (C18:1), Elaidic acid (*trans*-C18:1), Polyunsaturated fatty acids; Linoleic acid (C18:2), Linolenic acid (C18:3). The pK_a values of fatty acids are: Capric acid (~ 4.9 , Ref. [15]); Lauric acid (~ 5.3 , Ref. [16]); Oleic acid (~ 9.8 , Ref. [17]); Elaidic acid (~ 9.9 , Ref. [17]); Linoleic acid (~ 9.2 , Ref. [17]); and α -Linolenic acid (~ 8.3 , Ref. [17]). Cx:y is defined such that x is the number of carbons in the primary alkyl chain and y is the number of degrees of unsaturation.

Compared to fatty acids, monoglycerides bear the distinction of not having ionizable functional groups across relevant pH conditions, and hence are nonionic molecules with neutral electrical charge properties and some degree of polarity.

Antimicrobial lipids are classified based on their chain lengths and degrees of unsaturation. In biological systems, fatty acids typically possess an even number of carbon atoms between 4 and 28, although other odd-numbered versions are possible in select systems or synthetically produced. Fatty acids that less than 8 carbon atoms are known as short-chain, while those with greater than 12 are long-chain fatty acids and medium-chain fatty acids have between 8 and 12 carbon atoms [8, 18, 19]. Another important parameter is the number of degrees of unsaturation. In saturated fatty acids, all the carbon atoms are linked by single covalent bonds, while unsaturated fatty acids have one or more double bonds (degrees of unsaturation) in the carbon backbone. Specifically, unsaturated fatty acids having more than one double bond are identified as polyunsaturated fatty acids. The presence of double bonds, including the number of them and their orientation (*cis*- or *trans*-) can lead to significantly different physicochemical properties of fatty acids, even those having the same hydrocarbon length. Thus, classifying fatty acids and their derivatives is an important part of investigating trends in antibacterial activity with respect to molecular structure and shape. To this end, extensive microbiological studies have been conducted and some trends have been established. In general, unsaturated fatty acids have greater potency to inhibit bacterial growth than saturated ones with the same length of hydrocarbon chain [20-22]. Of note, unsaturated fatty acids with medium and longer chains typically show greater efficacy against Gram-positive bacteria than Gram-negative bacteria [23, 24]. Some studies focused on assessing antibacterial potency in the presence of compounds with double bonds and related properties [22, 24-27]. Of particular relevance to this thesis, the potency for saturated fatty acids has been examined as function hydrocarbon chain length [20, 24, 27-30]. By systematically investigating saturated fatty acids with hydrocarbon chains ranging between 6 and 18 carbons long, it was identified that lauric acid with a 12-carbon long chain has the most potent activity to inhibit growth of Gram-positive bacteria, including *Staphylococcus aureus*, a major causative agent of systemic and skin bacterial infections. Its 1-monoglyceride derivative called glycerol monolaurate (GML) showed

greater potency against *S. aureus*, as indicated by a lower value of minimum inhibitory concentration in comparison to lauric acid [20]. In addition, capric acid and its monoglyceride derivative, monocaprin, have saturated hydrocarbon chains that are 10 carbons long, and also have high antibacterial activity, especially against Gram-negative bacteria that are commonly associated with foodborne infections such as *Campylobacter jejuni* [31]. Of note, all four compounds are widely abundant in nature and Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration as food additives [7]. Motivated by the high reported antibacterial activity and other related factors, these four compounds were selected as the main ones for testing in this thesis along with appropriate controls where applicable.

2.1.3 Spectrum of Antibacterial Activity

Broad-spectrum inhibitory activity of antimicrobial lipids against algae, bacteria, fungi, protozoa, and virus has been reported for several decades [8, 12, 20, 32-35]. Of particular relevance to this thesis, this section focuses on describing the antibacterial activities of these compounds in detail, outlining the basic relationship between structure and function. The antibacterial potency of fatty acids and monoglyceride derivatives has been investigated extensively on a wide range of bacteria, including pathogenic strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), as presented in **Table 2.1**. Extensive screening of the antibacterial activities of fatty acids was conducted by Kabara and colleagues in the 1970's, and helped to establish the modern-day field of antimicrobial lipids from a chemical perspective. Fatty acids with hydrocarbon chains ranging from 6 to 18 carbons long and selected derivatives with different chemically functionalized headgroups were evaluated, resulting in the comprehensive identification of lauric acid (C12:0) as the most potent antimicrobial lipid to inhibit growth of Gram-positive bacteria. As a general principle the esterification of fatty acid to the corresponding monoglyceride derivative increases antibacterial activity. For example, the monoglyceride derivative of lauric acid, GML (MG C12:0), has greater potency than that of lauric acid, with a lower minimum inhibitory concentration (MIC) value as mentioned above [20, 32, 36]. Another supportive study for the high antibacterial potency of lauric

acid was conducted with different types of Gram-positive bacteria, and demonstrated that unsaturated fatty acids with 18 carbon long chains – oleic acid (C18:1), linoleic (C18:2), and linolenic acid (C18:3) – have potent antibacterial activity [24]. Based on the findings from these pioneering studies, more selective and detailed studies focused on either specific bacteria type or particular antimicrobial lipids have been performed [21, 22, 26, 37]. Specifically, interest on fatty acids and monoglycerides as potential therapeutic agents and/or preservatives against medically important pathogens led to the following studies of biomedical and food science relevance. Wang *et al.* investigated the efficacy of fatty acids and monoglycerides against *Listeria monocytogenes*, which is a Gram-positive bacterium that causes a series of food-borne infections. Of the tested fatty acids and monoglycerides, lauric acid, linolenic acid, and GML had the strongest bactericidal activity at 10-20 µg/mL in brain heart infusion broth at pH 5. Interestingly, the bactericidal activity of the fatty acids depended on solution pH, showing increased activity at pH 5 as compared to pH 6, while the activity of the monoglyceride was not influenced by solution pH across this range [38]. Another target bacterium that is susceptible to fatty acids is *Helicobacter pylori*, which is a Gram-negative bacterium that can be pathogenic in the stomach or duodenum leading to diseases such as chronic gastritis, gastric ulcers, and stomach cancer. Petschow *et al.* tested a wide range of saturated fatty acids and corresponding monoglycerides with chain lengths from 4 to 17 carbons against *H. pylori* and observed the following bactericidal activities. Monoglycerides with 10-14 carbon long chains showed appreciable bactericidal efficacy at 1 mM concentration and had a lower tendency of spontaneous resistance development, while lauric acid was the only saturated fatty acid that had bactericidal potency under the tested conditions [39]. Similarly, the anti-*H. pylori* activity of lauric acid and GML was demonstrated by Sun *et al.*, in which case selected unsaturated fatty acids were evaluated along with a range of saturated fatty acids (4-16 carbon chains) and monoglycerides (12-16 carbon chains). Additionally, it was identified that, of unsaturated fatty acids, myristoleic (C14:1) and linolenic acid (C18:3), have the most potent activity against this bacterium [29]. Bergsson and colleagues also assessed inhibitory action against Gram-negative bacteria, including *H. pylori*, for a wide range of fatty acids and corresponding monoglycerides [40]. Interestingly, the tested compounds that had hydrocarbon chains

between 10-16 carbons long exhibited inactivation of *H. pylori* at 10 mM concentration, however, there was no significant activity observed against *Salmonella* species and *E. coli*. Bergsson *et al.* conducted additional studies in order to characterize the antibacterial potency of selected antimicrobial lipids against different bacteria, and it was demonstrated that monocaprin (MG C10:0) is the most potent bactericidal agent against *Chlamydia trachomatis* and *Neisseria gonorrhoeae* [41, 42]. Additional studies have been reported investigating the effects of fatty acids (with chains of 2 to 18 carbon length) on Gram-negative bacteria, including *E. coli* [43] and *Salmonella* species [44], and demonstrated that caprylic acid (C8:0) has particularly high antibacterial activity against these bacteria. In this thesis, the model bacterium was *S. aureus* and methicillin-resistant *S. aureus* (MRSA) is a leading cause of skin and soft-tissue infections, including acute bacterial skin and skin structure infections (ABSSSIs). Within this scope, Kitahara *et al.* investigated a range of saturated fatty acids against conventional *S. aureus*, Methicillin-Susceptible *S. aureus* (MSSA) and MRSA, and it was identified that lauric acid is the most potent saturated fatty acid against MSSA and MRSA [45]. Towards developing therapeutics, Nakatsuji *et al.* explored the feasibility of employing lauric acid to treat a mouse model infection caused by a Gram-positive bacterium, specifically *Propionibacterium acnes* [46].

Table 2.1 Antibacterial activity of fatty acids and monoglycerides against different bacterial strains.

Bacteria	Fatty Acid [FAs] /Monoglycerides [MGs] *	Key Findings	Ref.
<i>Bacillus megaterium</i> (+) <i>Bacillus mycoides</i> (+) <i>Bacillus subtilis</i> (+) <i>Bacillus</i> sp. (+) <i>Streptococcus faecium</i> (+) <i>Streptococcus lactis</i> (+) <i>Staphylococcus</i> sp. (+) <i>Micrococcus</i> sp. (+) <i>Micrococcus lysodeikticus</i> (+) <i>Clostridium butyricum</i> (+) <i>Clostridium sporogenes</i> (+) <i>Clostridium welchii</i> (+)	[FAs [†]]: C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, <i>trans</i> -C18:1, C18:2, C18:3	Among saturated fatty acids, lauric acid (C12:0) was the most potent. Unsaturated fatty acids, linoleic (C18:2) and linolenic acid (C18:3), were more potent than lauric acid against <i>B. megaterium</i> .	[24]
Pneumococci (+) Streptococcus group A (+) Streptococcus beta- hemolytic non-A (+) <i>Corynebacterium</i> sp. (+) <i>Nocardia asteroides</i> (+) <i>Micrococcus</i> sp. (+) <i>Staphylococcus aureus</i> (+) <i>Staphylococcus epidermidis</i> (+) Streptococcus group D (+)	[FAs]: C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C14:1, C16:1, C18:1, <i>trans</i> -C18:1, C18:2, <i>trans</i> -C18:2, C18:3, C20:4 [MGs [‡]]: C10:0, C12:0	Lauric acid (C12:0) was the most potent saturated fatty acids against Gram-positive bacteria. Monocaprin (MG C10:0) and GML (MG C12:0) had greater antibacterial activity than fatty acid equivalents. GML had more potent activity with a lower MIC value than lauric acid against most Gram-positive bacteria.	[20]
<i>Streptococcus faecalis</i> (Group D) (+) <i>Streptococcus pyogenes</i> (+) <i>Staphylococcus aureus</i> (+) <i>Corynebacterium</i> sp. (+) <i>Nocardia asteroides</i> (+)	[FAs]: C11:0, C12:0, C13:0 [MGs]: C11:0, C12:0, C13:0	Lauric acid (C12:0) and GML (MG C12:0) were the most potent antibacterial compounds among those tested against Gram-positive bacteria. Esterification of fatty acids to monoglyceride form generally increased antibacterial activity.	[32]
<i>Mycobacterium smegmatis</i> (+)	[FAs]: C10:0, C12:0, C14:0, C16:0, C18:0, C20:0, C16:1, C18:1, C18:2, C20:4	Most unsaturated fatty acids showed potent bactericidal effect against <i>M. smegmatis</i> . Among saturated fatty acids, only lauric acid (C12:0) and myristic acid (C14:0) showed some degree of antibacterial activity at 0.2 mM concentration.	[37]

Table 2.1 *Cont.*

Bacteria	Fatty Acid [FAs] /Monoglycerides [MGs] *	Key Findings	Ref.
<i>Haemophilus influenzae</i> (-) <i>Neisseria gonorrhoeae</i> (-) <i>Escherichia coli</i> (-) <i>Staphylococcus aureus</i> (+) <i>Lactobacillus acidophilus</i> (+) <i>Bacillus megaterium</i> (+)	[FA] : C20:4	<p>All tested Gram-positive species were susceptible to treatment with 0.01 mM arachidonic acid.</p> <p><i>L. acidophilus</i> was most susceptible among Gram-positive bacteria.</p> <p>Bactericidal effect of arachidonic acid treatment on <i>S. aureus</i> depended on treatment time and drug concentration.</p>	[26]
<i>Listeria monocytogenes</i> (+)	[FAs] : C12:0, C14:0, C16:0, C18:0, C18:1, C18:2, C18:3 [MGs] C12:0, C14:0, C16:0	<p>Lauric acid (C12:0), linolenic acid (C18:3), and GML (MG C12:0) exhibited strong antibacterial activity against <i>L. monocytogenes</i> at 10-20 µg/ml.</p> <p>Bactericidal activity of lauric acid and linolenic acid in brain heart infusion broth was higher at pH 5 than pH 6.</p>	[38]
<i>Bacillus larvae</i> (+)	[FAs] : C10:0, C11:0, C12:0, C13:0, C14:1, C16:1, C18:2, etc.	<p>Lauric acid (C12:0) and myristoleic acid (C14:1) showed most potent activity against <i>B. larvae</i>.</p> <p>Saturated fatty acids with greater than 14- carbon long chains did not inhibit bacterial growth.</p>	[22]
<i>Helicobacter pylori</i> (-)	[FAs] : C4:0, C5:0, C6:0, C7:0, C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C12:1 [MGs] : C4:0, C5:0, C6:0, C7:0, C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C12:1	<p>Saturated monoglycerides with 10-14 carbon long chains showed bactericidal activity against <i>H. pylori</i> with more than 4 log₁₀ reduction upon treatment with 1 mM compound.</p> <p>Lauric acid (C12:0) was unique among tested saturated medium-chain fatty acids, to have antibacterial activity.</p> <p>Medium-chain monoglycerides inactivated <i>H. pylori</i> effectively and there was less spontaneous resistance development.</p>	[39]

Table 2.1 *Cont.*

Bacteria	Fatty Acid [FAs] /Monoglycerides [MGs] *	Key Findings	Ref.
<i>Chlamydia trachomatis</i> (-)	[FAs] : C8:0, C10:0, C12:0, C14:0, C16:1, C18:1 [MGs] : C8:0, C10:0, C12:0, C16:1, C18:1	Lauric acid (C12:0), capric acid (C10:0), and monocaprin (MG C10:0) had antibacterial activity against <i>C. trachomatis</i> upon treatment with 20 mM compound. Monocaprin was most potent to kill <i>C. trachomatis</i> , as suggested by destabilization of the bacterial membrane of elementary bodies.	[41]
<i>Neisseria gonorrhoeae</i> (-)	[FAs] : C8:0, C10:0, C12:0, C14:0, C16:1, C18:1 [MGs] : C8:0, C10:0, C12:0, C14:0, C16:1, C18:1	Monocaprin (MG C10:0) was the most potent to effectively kill <i>N. gonorrhoeae</i> . Lauric acid (C12:0) and palmitoleic acid (C16:1) had bactericidal activity against <i>N. gonorrhoeae</i> , as determined by treatment with 2.5 mM test compound.	[42]
<i>Helicobacter pylori</i> (-) <i>Salmonella</i> spp. (-) <i>Escherichia coli</i> (-)	[FAs] : C8:0, C10:0, C12:0, C14:0, C16:1, C18:1 [MGs] : C8:0, C10:0, C12:0, C14:0, C16:1, C18:1	Tested compounds with 10-16 carbon long chains were active against <i>H. pylori</i> , while largely inactive against <i>Salmonella</i> spp. and <i>E. coli</i> . Monocaprin (MG C10:0) and GML (MG C12:0) showed highest levels of inhibitory activity against <i>H. pylori</i> .	[40]
<i>Helicobacter pylori</i> (-)	[FAs] : C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C14:1, C16:1, C18:1, C18:2, C18:3 [MGs] : C12:0, C14:0, C16:0	Among saturated fatty acids, lauric acid (C12:0) was the most potent bactericidal compound against <i>H. pylori</i> , with an MBC value of 1 mM. GML (MG C12:0) was the most potent monoglyceride and had a lower MBC value than lauric acid. Among unsaturated fatty acids, myristoleic (C14:1) and linolenic acid (C18:3) had the most potent antibacterial activity.	[29]

Table 2.1 Cont.

Bacteria	Fatty Acid [FAs] /Monoglycerides [MGs] *	Key Findings	Ref.
<i>Escherichia coli</i> (-)	[FAs] : C2:0, C3:0, C4:0, C5:0, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, C18:2	Caprylic (C8:0) and capric acid (C10:0) showed antibacterial activity against <i>E. coli</i> ; caprylic acid had highest activity. Bactericidal effect of the two fatty acids was higher at pH 5.2.	[43]
<i>Salmonella enteritidis</i> (-) <i>Salmonella infantis</i> (-) <i>Salmonella typhimurium</i> (-)	[FAs] : C2:0, C3:0, C4:0, C5:0, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, C18:2	Caprylic acid (C8:0) alone showed antibacterial activity against <i>Salmonella</i> strains under lower pH conditions around 5.2-5.3.	[44]
<i>Staphylococcus aureus</i> (+) Methicillin-Susceptible <i>Staphylococcus aureus</i> (MSSA) (+) Methicillin-Resistant <i>Staphylococcus aureus</i> (MRSA) (+)	[FAs] : C8:0, C10:0, C12:0, C14:0, C16:0, C18:0	Lauric acid (C12:0) was most potent saturated fatty acids against MSSA and MRSA strains and inhibit their growth at 400 mg/mL test concentration.	[45]
<i>Clostridium perfringens</i> (+)	[FAs] : C2:0, C3:0, C4:0, C5:0, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, C18:2	Lauric acid (C12:0) was the most potent fatty acids against <i>C. perfringens</i> and maintained its activity at pH > 6, while capric acid was only active at pH 5.0-5.3.	[47]
<i>Lactococcus garvieae</i> (+) <i>Vibrio harveyi</i> (-) <i>Vibrio anguillarum</i> (-) <i>Vibrio alginocolyticus</i> (-)	[FAs] : C15:0, C16:0, C17:0, C18:0, C22:0, C18:1, C18:4, C20:4, C20:5, C22:4, C22:5	Unsaturated fatty acids showed greater bactericidal effect on tested bacteria than saturated fatty acids.	[48]
<i>Propionibacterium acnes</i> (+) <i>Staphylococcus aureus</i> (+) <i>Staphylococcus epidermidis</i> (+)	[FAs] : C12:0	Lauric acid (C12:0) had greater antibacterial activity against <i>P. acnes</i> than benzoyl peroxide (BPO). Lauric acid was not cytotoxic against human sebocytes.	[46]
<i>Staphylococcus aureus</i> (+) <i>Streptococcus pyogenes</i> (+)	[FAs] : C12:0 [MGs] : C12:0	GML (MG C12:0) had 200-fold greater bactericidal potency than lauric acid (C12:0) against <i>S. aureus</i> .	[49]

* [number of carbon atoms in alkyl chain:number of double bonds]

† FA indicates 'fatty acid'.

* MG indicates 'monoglyceride'.

2.1.4 Mechanisms of Antimicrobial Activity

The mechanism(s) of antimicrobial activity of fatty acids and monoglycerides have been explored using diverse experimental methods to identify that they mainly target bacterial cell membranes and interrupt crucial processes involved in cellular protection and function. The membrane-lytic behavior of fatty acids and monoglycerides stem from their amphipathic properties, leading to overlapping sets of biophysical phenomena including membrane destabilization and transient or permanent pore formation. In particular, membrane-destabilizing activity causes increased cell permeability and cell lysis, leading to inhibition of bacterial cell growth (bacteriostatic action) or cell death (bactericidal action). Among vital processes involving bacterial cell membranes, two of the most important ones involve the electron transport chain and oxidative phosphorylation, which are essential for energy production in bacterial cells. The two processes are connected and interact with one another, and fatty acids have the potential to disrupt the electron transport chain process by binding to electron carriers or altering membrane integrity as well as interfere with oxidative phosphorylation by decreasing the membrane potential and proton gradient. Moreover, fatty acids can directly inhibit membrane enzymes such as glucosyltransferase, presumably due to similar molecular structures of fatty acids with known small molecule inhibitors [50, 51]. **Figure 2.2** presents a schematic illustration describing key antibacterial activities of fatty acids and monoglycerides that relate to targeting bacterial cell membranes. The details of the mechanistic processes can be classified based on the relationship between the following three aspects: (i) increased membrane permeability and cell lysis, (ii) disruption of electron transport chain and uncoupling oxidative phosphorylation, and (iii) inhibition of membrane enzymatic activities and nutrient uptake.

Figure 2.2 Schematic representation of mechanisms behind the antibacterial activity of fatty acids and monoglycerides.

Increased membrane permeability and cell lysis

The interaction of antimicrobial lipids with bacterial cell membranes can destabilize the membrane and increase membrane permeability, thereby inducing leakage of cytosolic contents. In extreme cases, the increased permeability and corresponding membrane destabilization can eventually lead to cell lysis, as documented in numerous experimental studies. For example, Chamberlain *et al.* observed that when *S. aureus* cell membranes were treated with oleic acid, there was an increase in membrane permeability as determined by fluorescence polarization. Oleic acid treatment lowered the polarization value, which caused increased membrane fluidity and led to cell death [52]. Similarly, linolenic acid treatment caused the damage of *S. aureus* cell membranes as reported by Greenway *et al.* In that case, membrane leakage after linolenic acid treatment was detected by measuring the release of biomolecules (*e.g.*, glutamic acid) from bacteria, and absorbance spectroscopy experiments at the 260 nm wavelength were conducted to measure the release which is correlated with the extent of bacterial cell membrane leakage [53]. As such, the measurements showed that linoleic acid inhibits the growth of

S. aureus by inducing a marked increase in membrane permeability — interpreted as pore formation – and this increased permeability further inhibits macromolecular synthesis and coupling of the electron transfer chain [21]. Boyaval *et al.* also demonstrated that the strong membrane-disruptive activity of linoleic acid causes leakage against *Propionibacterium freudenreichii* subsp *shermanii*. In particular, linoleic acid interrupted bacterial cell growth by increasing membrane permeability, as measured by monitoring potassium efflux and transmembrane electrical potential. Increased permeability resulted in an increase in K^+ efflux and a decrease in membrane potential [54]. Beyond biochemical readouts, many studies have reported using electron microscopy to investigate the membrane-disruptive activity of fatty acids against various bacterial cell types [26, 38, 41, 47, 55, 56]. In most of these studies, high concentrations of antimicrobial lipid in the millimolar range were used and hence the membrane morphological damage was quite extensive and observed post-treatment. In addition, the membrane-disruptive activities of monoglycerides against different bacterial cell types have been reported [38, 41]. (details of references reporting antibacterial activities against cell membrane targets will be further discussed in Section 2.2.1.3) Regarding direct observation of cell lysis, Carson *et al.* reported that certain unsaturated fatty acids, namely oleic acid or linoleic acid, can cause lysis of *Streptococcus faecalis* [57]. Thompson *et al.* further showed how treatment of *H. pylori* with linolenic acid induces cell lysis, as revealed by electron microscopy [58].

Disrupting electron transport chain and uncoupling oxidative phosphorylation

The electron transport chain is a key complex that consists of electron carriers and produces the energy source, adenosine triphosphate (ATP), and is coupled to oxidative phosphorylation through the ATP synthase, an enzyme that synthesizes ATP. The electron transport chain and ATP synthase are both located at the inner membrane of Gram-positive and Gram-negative bacteria [59]. Electrons are transported from one carrier to another until reaching the final electron acceptor, which is oxygen [60]. The electron transport process is accompanied by proton (H^+) transfer from the cytosol to outside of the cell, creating a proton gradient across the cytoplasmic membrane and increasing membrane potential that provides an energy source to produce ATP *via* the

ATP synthase. When one of the steps in the electron transport chain and oxidative phosphorylation are interrupted, it is difficult for bacterial cells to have sufficient energy to function, which leads to inhibition of cell growth and eventually cell death. By measuring oxygen uptake, interruption of the electron transport chain can be assessed. Following this approach, Galbraith *et al.* demonstrated that lauric acid and myristic acid were the most effective saturated fatty acids to inhibit oxygen intake for *Bacillus megaterium* and *Pseudomonas phaseolicola*, while linoleic acid was the most effective unsaturated fatty acid and was active at much lower concentrations (greater potency) than saturated fatty acids. More specifically, linoleic acid induced significant decrease of oxygen uptake in target bacteria at 0.1 mM compared to lauric acid having similar effect at 1 mM treatment [61]. In another related study, Greenway *et al.* investigated the disruption of the electron transport chain in *S. aureus* in response to linoleic acid treatment [21]. Moreover, Sheu *et al.* investigated the effect of fatty acid treatment on *Bacillus subtilis* by measuring oxygen uptake and ATP concentrations, and demonstrated that the interaction of fatty acids with the bacterial cell membrane changed membrane integrity, resulting in decreases in oxygen uptake and ATP levels [62]. While it is difficult to directly measure the effect of fatty acid treatment on electron carrier transport in live bacteria, it is noteworthy that Peters *et al.* investigated electron transport in intact chloroplasts, demonstrating that palmitoleic acid (C16:1 fatty acid) mainly inhibited photosystem II by up to 90% as part of the electron transport system. Although the study was not conducted on bacterial cell membranes, the results still provide useful insight to understand how membrane destabilization caused by antimicrobial lipids can cause severe detriments to electron transport carriers and downstream biochemical processes [63].

Inhibiting activity of bacterial enzymes

Another significant mechanism by which antimicrobial lipids affect bacterial cell membranes is inhibiting the activity of membrane-associated enzymes, and there have been numerous studies exploring the effects of fatty acid treatment on specific bacterial cell enzymes. For example, Kurihara *et al.* observed that certain fatty acids inhibit glucan production catalyzed by glucosyltransferase (GTase) from *Streptococcus sobrinus*, which

leads inhibition of bacterial cell growth. GTase is an important transmembrane protein that mediates glucan production in bacterial cells and is mainly produced by *Streptococcus mutans* and *S. sobrinus*. Significantly, unsaturated fatty acids such as oleic acid (C18:1), linoleic acid (C18:2) and arachidonic acid (C20:4) exhibit even stronger inhibition of GTase activity, while there is an almost negligible effect caused by saturated fatty acids [50]. This finding is significant because it supports that antimicrobial lipids with different physicochemical properties can have unique modes of interacting with bacterial cell membranes. Another study also showed GTase inhibition by oleic acid, supporting that the antibacterial activity of unsaturated fatty acids is related, at least in part, to inhibiting membrane-associated enzymes [51].

Additionally, it has been reported that fatty acids and related derivatives can inhibit bacterial growth by inhibiting fatty acid biosynthesis. Indeed, fatty acids play important roles in bacteria because they are precursors of important cellular materials. Zheng *et al.* demonstrated that unsaturated fatty acids, including linoleic acid, show antibacterial activity against *S. aureus* by inhibiting bacterial enoyl-acyl carrier protein reductase (FabI), which is an important enzyme involved in the fatty acid elongation process [64]. Similarly, the antibacterial activity of medium-chain saturated and unsaturated fatty acids against *S. aureus* was investigated in terms of inhibiting fatty acid synthesis, and the results indicated that, among the tested compounds, α -linolenic acid was particularly inhibitory [65]. To what extent the effects of antimicrobial lipids on bacterial enzymes are direct or indirect remain to be understood in the broader context of membrane destabilization processes, while it is clear that the effects of antimicrobial lipids on bacterial membranes can inhibit key enzymatic activities. Within this scope, it is particularly intriguing that unsaturated fatty acids affect bacterial enzymes, while saturated fatty acids typically have negligible effect on the same enzymes. Such findings motivate the overall motivation of this thesis to establish measurement platforms for characterizing the mechanism of action and potency of antimicrobial lipids acting against phospholipid membranes, and to draw correlations with biological activities. In the following section, the main experimental techniques to characterize antimicrobial lipids are presented, including microbiological and biophysical method.

2.2 Experimental Approaches to Characterize Antimicrobial Lipids

The inhibitory activity of antimicrobial lipids has been widely investigated by employing biological approaches based on anti-infective evaluation of bacterial specimens. Antibacterial susceptibility tests such as MIC and minimum bactericidal concentration (MBC) assays are commonly used. The MIC assay is a method of determining the minimum concentration of a test compound that inhibits bacterial growth, thereby enabling rapid screening of the antibacterial susceptibility of antimicrobial lipids against a target bacterium. A more detailed understanding about the mode of action of a test compound can be obtained by determining the MBC value, which is defined as the minimum concentration of a test compound to completely kill a target bacterium. Although both MIC and MBC assays facilitate empirical evaluation of the antibacterial potency of a drug candidate, and inform about the antibacterial spectrum of an antimicrobial lipid, the assays do not probe how antibacterial lipids destabilize bacterial cell membranes. To address such questions, electron microscopy has been widely used to visualize the antibacterial activity of antimicrobial lipids by observing morphological changes of bacterial specimens after treatment with antimicrobial lipids. While useful to look at gross morphological changes, this approach has limitations because it typically requires high lipid concentrations and the bacterial cells are analyzed after treatment and sample fixation. To facilitate real-time monitoring of the membrane interactions involving antimicrobial lipids, one of the most useful approaches has focused on developing model membrane platforms to investigate molecular-level interactions. The different experimental techniques used in biological and biophysical studies are summarized in **Table A.1**. Indeed, the measurement approach is broadly applicable to studying how environmental factors such as temperature and solution pH affect the properties of antimicrobial lipids, with respect to physicochemical changes in lipid structure along with membrane characteristics.

2.2.1 Anti-Infective Evaluation of Bacterial Specimens

2.2.1.1 Growth Inhibition Assays

One of the most widely used experimental assays to determine the antibacterial activity of test compounds involves determining the minimum inhibitory concentration (MIC) of a compound that is able to inhibit bacterial growth. Formally, the MIC is defined as the lowest concentration of test compound that inhibits observable bacterial growth, and MIC values are often used as quantitative indicators of the relative potency of new antibacterial agents [66]. The agar and broth dilution methods are the most common protocols for determining MIC values. Briefly, in the agar method, a certain number of bacteria are directly added onto agar plates containing one specific antibacterial compound, and this approach is advantageous to evaluate many types of bacteria at the same time under identical conditions. In contrast, the broth dilution method utilizes a liquid medium containing serial concentrations of the antibacterial agent, and is particularly effective for examining a large number of bacterial species across multiple test compounds. Depending on the volume of the test sample, the broth dilution method can be classified as either broth macrodilution or microdilution. Both agar dilution and broth macrodilution methods require a large amount of test compound, and thus the broth microdilution is defined as a broth dilution that is performed by using microtiter plates with maximum volume of 500 μ l per well. The latter method is commonly used for determining MIC values of fatty acids and monoglycerides in recent years [67, 68]. The results obtained by conducting broth microdilution experiments are mainly defined by the MIC value, which is recorded by assessing optical turbidity visually or with a microplate reader. However, the MIC values obtained for the same compound can often be quite variable depending on different parameter, *e.g.*, broth or buffer composition and bacterial cell density. For example, using the same measurement method, it has been reported that MIC value of LA against *S. aureus* varied around 500-1000 μ M and GML around 31-125 μ M depending on the preparation method in PBS solution or Mueller-Hinton (MH) Broth [69]. Even greater variations are reported in the literature when considering variations in experimental method. Additionally,

antimicrobial lipids are particularly sensitive to temperature [70, 71] and pH [29, 37, 38, 43, 44, 47, 49, 72, 73] as well, the experimental conditions should be designed and modified appropriately depending on the type of test compound.

To date, MIC studies are one of the most common methods to study antimicrobial lipids. In the 1970's, Kabara and colleagues conducted pioneering studies to evaluate the MIC values of fatty acids and monoglycerides against a wide range of bacteria by using the broth dilution method, and the recorded MIC values are summarized in **Tables 2.2** and **2.3** for fatty acids and monoglycerides, respectively. In addition, Galbraith and Nakatsuji also tested fatty acids against other bacteria of interest. Of note, Kitahara *et al.* reported a unconventional method to determine MIC values based on measuring oxygen levels using an oxygen electrode sensor named DOX-96 [45]. As mentioned above, saturated fatty acids and monoglycerides with 12-carbon long chains exhibited the most potent activity to inhibit the growth of Gram-positive bacteria, with particularly high antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strain which causes serious acute skin infections in humans [74]. In general, monoglycerides have lower MIC values than fatty acid equivalents against different bacteria. Sometimes, reported MIC values are quite different for the same test compound against the same bacteria, due to variations in experimental conditions. For examples, multiple reports by Kabara and colleagues showed similar MIC values for the same compound (prepared in roughly equivalent ways in the two studies) against *S. aureus*, as determined by the broth dilution method [20, 32]. In contrast, comparing the MIC values obtained for lauric acid (C12:0) against *S. aureus* in studies by different groups reveals that the potency of a single compound against a single bacterium can vary by over 100-fold. In the two studies, it was noticed that different organic solvents were used for solubilizing lauric acid molecules, and the highest test concentrations contained 1% ethanol or 5% dimethyl sulfoxide (DMSO) in the two studies, respectively. As such, the presence of organic solvents or other environmental factors likely influences the molecular self-assembly of antimicrobial lipids in bulk solution, and such variations are only reflected in the MIC readout on the basis of how the compounds inhibits bacterial growth. For this reason, MIC assays provide an initial empirical assessment of antibacterial activity but do not

yield detailed information about the mechanism of action of a compound or offer insight into how the potency of a compound might be optimized.

Table 2.2 Selected MIC values of fatty acids against different bacteria.

Bacteria	Fatty Acids (number of carbon atoms in alkyl chain:number of double bonds)								Ref.
	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3	
<i>B. megaterium</i>	1.0 mM	0.15 mM	0.15 mM	0.3 mM	0.4 mM	0.05 mM	0.02 mM	0.02 mM	[24]
Pneumococci	1.45 mM	0.062 mM	0.218 mM	0.48 mM	NI [†]	NI	0.044 mM	0.179 mM	[20]
Streptococcus group A	1.45 mM	0.124 mM	0.547 mM	3.9 mM	NI	1.77 mM	0.089 mM	0.35 mM	
Streptococcus group D	5.8 mM	2.49 mM	4.37 mM	NI	NI	NI	NI	NI	
Streptococcus beta-hemolyticnon-A	2.9 mM	0.249 mM	2.18 mM	3.9 mM	NI	NI	0.089 mM	0.35 mM	
<i>Micrococcus</i> sp.	2.9 mM	0.624 mM	0.547 mM	1.9 mM	NI	NI	0.089 mM	0.488 mM	
<i>Corynebacterium</i> sp.	1.45 mM	0.124 mM	0.437 mM	1.9 mM	NI	NI	0.044 mM	0.179 mM	[32]
	--	31 µg/ml	--	--	--	--	--	--	
<i>N. asteroides</i>	1.45 mM	0.124 mM	0.547 mM	NI	NI	NI	0.089 mM	0.448 mM	[20]
	--	62 µg/ml	--	--	--	--	--	--	[32]
<i>S. epidermidis</i>	2.9 mM	2.49 mM	2.18 mM	3.9 mM	NI	NI	NI	NI	[20]
	--	3.9 µg/ml	--	--	--	--	--	--	[46]
<i>S. aureus</i>	2.9 mM	2.49 mM	4.37 mM	NI	NI	NI	NI	1.79 mM	[20]
	--	500 µg/ml	--	--	--	--	--	--	[32]
	--	0.97 µg/ml	--	--	--	--	--	--	[46]
MSSA *	800 µg/ml	400 µg/ml	1600 µg/ml	> 1600 µg/ml	> 1600 µg/ml	--	--	--	[45]
MRSA	800 µg/ml	400 µg/ml	1600 µg/ml	> 1600 µg/ml	> 1600 µg/ml	--	--	--	
<i>Strep. faecalis</i>	--	500 µg/ml	--	--	--	--	--	--	[32]
<i>Strep. pyogenes</i>	--	62 µg/ml	--	--	--	--	--	--	
<i>P. acnes</i>	--	3.9 µg/ml	--	--	--	--	--	--	[46]

* Methicillin-susceptible *Staphylococcus aureus*.

[†] NI indicates no bacterial growth inhibition observed within the tested concentration range.

Table 2.3 Selected MIC values of monoglycerides against different bacteria.

Bacteria	Monoglycerides (number of carbon atoms in alkyl chain:number of double bonds)			Ref.
	C10:0	C12:0	C13:0	
Pneumococci	0.1 mM	0.09 mM	--	[20]
Streptococcus group A	0.2 mM	0.045 mM	--	
Streptococcus group D	2.0 mM	NI *	--	
<i>Micrococcus</i> sp.	0.1 mM	0.09 mM	--	
<i>S. epidermidis</i>	1.0 mM	0.09 mM	--	
<i>Corynebacterium</i> sp.	0.2 mM	0.045 mM	--	[32]
	--	16 µg/ml	NI	
<i>N. asteroides</i>	0.5 mM	0.09 mM	--	[20]
	--	16 µg/ml	125 µg/ml	[32]
<i>S. aureus</i>	1.0 mM	0.09 mM	--	[20]
	--	250 µg/ml	NI	[32]
<i>Strep. faecalis</i>	--	NI	NI	
<i>Strep. pyogenes</i>	--	8 µg/ml	62 µg/ml	

* NI indicates no bacterial growth inhibition observed within the tested concentration range.

2.2.1.2 Infectivity Assays

While MIC readouts assess the capacity of a drug candidate to inhibit bacterial growth, the information obtained does not provide direct information about whether or not treatment with a drug can directly inactivate a bacterium by way of killing, *e.g.*, lytic effect. To address such questions, which are particularly relevant to consider for antimicrobial lipids since they are membrane-lytic agents, other methods have been devised and involve determining the minimum bactericidal concentration (MBC), which is defined as the lowest concentration of antimicrobial agent to kill a target bacterium and can be obtained with a subcultures of broth MIC assay in drug-free agar media. Briefly, expanding on the MIC assay using the broth dilution method, after incubating a defined number of target bacteria (typical final inoculum size is 5×10^5 CFU/ml) with a range of test compounds for a fixed time period (often several hours), the incubated mixtures at each concentration are serially diluted in 10-fold series and then subcultured on drug-free agar medium. Colonies from the subculture agar plates are counted and quantified to colony-forming units (CFU) per ml, and the values obtained can be compared to those of control bacteria. Typically, the MBC value is the lowest concentration of a test compound at which $\geq 99.9\%$ ($\geq 3 \log_{10}$ CFU/ml reduction) of the initial bacterial inoculum are killed within 24 hrs [75]. Some studies have slightly modified this definition as $\geq 99.99\%$ reduction of the initial inoculum after a certain time of incubation [38]. Normally, if the determined MBC value is no more than 4 times greater than the MIC value of a test agent, then the agent is considered to have bactericidal activity. Otherwise, the candidate is considered to have principally bacteriostatic activity.

There are many studies investigating the bactericidal activity of fatty acids and monoglycerides by determining MBC values. Sun *et al.* reported the MBC values of the C12 saturated fatty acid and monoglyceride pair, lauric acid and GML, against *H. pylori*. The MBC values of lauric acid and GML were 1 mM and 0.5 mM, respectively [29]. Wang *et al.* tested lauric acid, linolenic acid, and GML and showed that all three compounds exhibit bactericidal effects against *L. monocytogenes*. Lauric acid, linolenic acid, and GML had MBC values of 10, 20, and 10 $\mu\text{g/ml}$, respectively, at the pH 5 condition. Interestingly, at higher pH conditions around 6, the MBC values of lauric acid

and linolenic acid increased to 20 and 100 $\mu\text{g/ml}$, respectively, while the MBC value of GML remained unchanged at 10 $\mu\text{g/ml}$ and was not influenced by the change in solution pH [38]. In addition, the MBC value of lauric acid against *P. acnes* was also determined to be 60 $\mu\text{g/ml}$ [46]. Direct comparison of MBC values for lauric acid and GML against *S. aureus* was also carried out, and it was reported that the MBC values for lauric acid and GML are 50 mM and 0.25 mM, respectively [49]. This finding indicated that GML has about a 200-fold lower MBC value and appreciably greater bactericidal activity against *S. aureus*. Without directly mentioning the MBC concept, Petschow *et al.* counted the number of viable bacterial cells by CFU plating after treatment with fatty acids or monoglycerides, and demonstrated that lauric acid is the only saturated fatty acid that shows bactericidal activity. In particular, treatment of *H. pylori* with 1 mM lauric acid for one hour yielded a greater than 4 $\log_{10}$ CFU/ml reduction. Among tested monoglycerides, monocaprin and GML also showed a bactericidal effect in similar fashion [39]. Although MBC is an excellent metric to evaluate antibacterial activity and help understand mechanistically whether or not a test compound completely kills or inhibits the growth of a bacterium, MBC values alone can also be variable depending on the technical format and experimental conditions, in analogous fashion to the challenges facing MIC determinations. Ultimately, both MIC and MBC values provide insight into the scope and potency by which antimicrobial lipids affect the infectivity of bacterial species, and can guide structure-activity relationships at the biological level. However, such assays do not provide information about how antimicrobial lipids destabilize bacterial cell membranes and hence there is limited room to explore optimization strategies or understand the physicochemical basis underpinning the scope and potency of particular compounds.

2.2.1.3 Electron Microscopy

Electron microscopy is a widely used class of measurement techniques for investigating the morphological structure of bacterial cell samples [76]. It has been utilized extensively for direct observation of antibacterial effects of fatty acids and monoglycerides acting against target bacterium. There are two main types of electron microscopy, referred to as scanning electron microscopy (SEM) and transmission

electron microscopy (TEM). SEM detects scattered electrons to visualize the sample surface, while TEM detects transmitted electrons that pass through the sample and thereby enables characterization of surface morphology along with the density of inner constituents [77]. To aid interpretation of cytoplasmic contents, samples for TEM measurements should be very thin and are prepared by sectioning [78].

When electron microscopy techniques were first introduced for studying antimicrobial lipids, most related studies utilized TEM for imaging the effects of treating bacteria with fatty acids. A summary of key observations made by electron microscopy analysis is reported in **Table 2.4**, including treatment conditions with antimicrobial lipid and corresponding target bacterium. In the late 1970's, Speert *et al.* explored the detailed mechanism of bactericidal activity underpinning how oleic acid affects Group A streptococci. Based on TEM measurements, it was observed that cytoplasmic contents become disorganized, including vacuolization in the cytosol and condensation of nucleoids [56]. Following this work, Knapp *et al.* observed significant membrane disruption of *N. gonorrhoeae* when the bacterium was treated with 10 μ M arachidonic acid, as revealed in TEM experiments [26]. All of the cytoplasmic contents appeared to leak out from morphologically deformed *N. gonorrhoeae* cells. In contrast, however, similar treatment did not affect the surface morphology of *S. aureus* cells, while there was still disruption and condensation of cytoplasmic contents in the latter case. There are also some TEM studies that investigate how monoglycerides affect bacterial cells. Based on extensive screening of the antibacterial activity of fatty acids and monoglycerides, Wang *et al.* identified that linolenic acid and GML are the most potent fatty acids and monoglycerides, respectively, against *L. monocytogenes* and the antibacterial effects were further investigated by TEM. Interestingly, cell lysis occurred upon treatment with 50 μ g/ml GML along with leakage of cytoplasmic contents, as presented in **Figure 2.3A,B**. On the other hand, upon treatment with 200 μ g/ml linolenic acid, only irregular changes in surface morphology were detected without cell lysis [38]. The antibacterial activity of monolaurin against bacterial cells has also been studied by Bergsson and colleagues. Upon treatment with 10 mM monolaurin, the elementary body form of *C. trachomatis* became shrunken through morphological deformation [41]. Subsequent studies exploring the potency of monolaurin were carried out on Group B streptococci, first by using SEM

followed by TEM experiments. Significant changes in surface morphology, including size and shape, were not observed by SEM, while TEM further revealed that the bacterial cell membrane and granule structures disappeared after monocaprin treatment, although the cell wall remained intact [28]. Similarly, lauric acid also disrupted and separated cell membranes in *C. perfringens* leading to cytoplasmic disorganization without causing significant changes in cell wall structure, as presented at **Figure 2.3C, D** [47]. Using SEM, Shin *et al.* showed that after treating *S. aureus* and *P. aeruginosa* with eicosapentaenoic acid, severe morphological disruption was observed with rough surface features becoming apparent [55].

Figure 2.3 TEM micrographs show the effect of treating bacterial cells with fatty acids and monoglycerides. *L. monocytogenes* cells that are (A) untreated or (B) treated with 50 µg/ml GML [38] and *C. perfringens* cells that are (C) untreated or (D) treated with 1 mg/ml lauric acid [47].

Table 2.4 Electron microscopy studies reporting how antibacterial fatty acids and monoglycerides affect bacterial cells.

Antimicrobial Lipid	Bacteria	Tech.	Key Observations	Ref.
Oleic acid (C18:1)	Group A streptococci (+)	TEM	Oleic acid aggregates attached around individual cells of group A streptococci and interacts with the bacterial cell membrane. Cytoplasmic changes occurred upon treatment, inducing vacuolization and nucleoid aggregation.	[56]
Arachidonic acid (C20:4)	<i>Neisseria gonorrhoeae</i> (-) <i>Staphylococcus aureus</i> (+)	TEM	Disruption of <i>N. gonorrhoeae</i> bacterial membrane was induced by 10 μ M arachidonic acid treatment, leading to morphological deformation and leakage of cytoplasmic contents. Similar treatment of <i>S. aureus</i> cells did not cause significant change of the cell wall structure, while condensation of cytoplasmic contents and other effects were observed.	[26]
Linolenic acid (C18:3)	<i>Listeria monocytogenes</i> (+)	TEM	Significant lysis of <i>L. monocytogenes</i> cells was observed upon treatment with 50 μ g/ml GML. Upon treatment with linolenic acid, cells exhibited irregular surface morphologies without apparent lysis.	[38]
GML (MG C12:0)				
Monocaprin (MG C10:0)	<i>Chlamydia trachomatis</i> (-)	TEM	<i>C. trachomatis</i> in elementary body form exhibited morphological deformations and shrunken shape upon treatment with 10 mM monocaprin.	[41]
Monocaprin (MG C10:0)	Group B streptococci (+)	TEM SEM	Upon treatment with 10 mM monocaprin, the surface morphology of Group B streptococci was unaffected, as determined by SEM. Disruption of plasma cell membrane and disappearance of granules were observed by TEM.	[28]
Lauric acid (C12:0)	<i>Clostridium perfringens</i> (+)	TEM	Upon treatment with 1 mg/ml lauric acid, complete cell membrane separation and disruption of <i>C. perfringens</i> cells were observed, but there was no structural change in the cell wall. Cytoplasmic contents became disordered upon treatment.	[47]
Eicosapentaenoic acid (EPA) (C20:5)	<i>Staphylococcus aureus</i> (+) <i>Pseudomonas aeruginosa</i> (-)	SEM	Upon treatment with EPA, morphological deformations along with the appearance of irregular bacterial surfaces were observed. Upon treatment, <i>S. aureus</i> cells lost regular shape and became rough, while hollow structures became evident on the surface on the surface of <i>P. aeruginosa</i> cells.	[55]

Taken together, a vast body of knowledge about how antimicrobial lipids affect bacterial cells has been elucidated by electron microscopy techniques. Among the findings, it is evident that there is a spectrum of ways in which antimicrobial lipids can perturb bacterial cells, including disruption of cell membranes and related effects such as loss of cytoplasmic contents. However, at the same time, distilling the empirical insights into general principles describing how antimicrobial lipids affect bacterial cell membranes remains difficult to achieve with electron microscopy results. Aside from the high lipid concentrations that are commonly tested, the low measurement throughput, and the divergent structural and compositional properties of different bacterial species, it is only possible to examine the effects of treatment after bacterial specimens have been fixed and therefore it is not possible to monitor changes in morphological properties in real-time. To address such needs, a wealth of biophysical techniques has been developed based on employing model membrane systems.

2.2.2 Biophysical Approaches with Model Membrane Platforms

2.2.2.1 Solution-Phase Liposomes

Solution-phase liposome assays have been developed to monitor the interaction kinetics between antimicrobial lipids and phospholipid membranes, by measuring changes in the size distribution of liposomes in bulk solution. One advantage of this approach is that the phospholipid compositions of the liposomes are highly simplified and therefore offer excellent control to understand how specific factors influence resulting interaction processes. Among the measurement options, dynamic light scattering is utilized as an ensemble-average measurement technique to determine the size and polydispersity of liposomes in bulk solution, including size changes after treatment with antimicrobial lipids. While the data is weighted by the intensity of individual liposomes that scatter light, some approximations can be applied to estimate the number of liposomes with different sizes as well. In addition, electron microscopy is employed to visualize how antimicrobial lipids induce morphological changes on individual liposomes. To date, several studies have been reported that perform complementary electron

microscopy and dynamic light scattering experiments and focused on oleic acid/oleate, as presented at **Table 2.5**. By using the thin film hydration method, liposomes can be prepared across a range of sizes, which are classified according to size as small unilamellar vesicle (SUVs) up to 100 nm diameter, large unilamellar vesicles (LUVs) with diameters between 100 and 400 nm [79], and giant unilamellar vesicles (GUVs) with diameters above 1 μm [80]. The studies were typically related to a “matrix effect” describing the phenomenon that, in presence of preexisting liposomes, the rate of forming new liposomes – as induced by the fatty acids – is appreciably accelerated, and the size distribution of the newly formed liposomes depended on the size of the preexisting liposomes and/or the molar ratio of fatty acids added to the liposomes [81, 82]. The first direct observation of the matrix effect was made when Blöchliger *et al.* showed that autocatalysis of oleate in aqueous solution was accelerated to form new liposomes in the presence of preexisting oleic acid/oleate liposomes [81]. In addition to the presence of the preexisting liposomes, the size distribution of newly formed mixed liposomes appeared within a narrow size range and the results showed a similar size distribution to the preexisting liposomes (*e.g.*, 50 or 100 nm diameter), while the newly formed liposomes from oleate itself without the preexisting liposomes had broad range of sizes ranging from 50 nm to 1.5 μm diameter. Of direct relevance to this thesis, there are numerous studies reporting the “matrix effect” when using phospholipid liposomes [82-88]. Lonchin *et al.* studied how different molar ratios of oleate added to preexisting POPC liposomes influences the size distribution of newly formed liposomes. Adding a large amount of oleate to POPC liposomes induced the formation of larger mixed POPC/oleate liposomes with increased dispersity, while similar sizes of mixed liposomes occurred when a lower amount was added [82]. Berclaz *et al.* provided further evidence showing how the size distribution of newly formed liposomes varies depending on the amount of oleate amount added to preexisting phospholipid liposomes, as determined by cryogenic TEM (cryo-TEM) analysis [86, 89]. Using DLS, Rasi *et al.* also investigated the formation of newly formed liposomes in response to the matrix effect caused by oleate treatment to POPC liposomes. The size distribution curves before (curve b) and after (curve c) oleate addition to preexisting 50 and 100 nm POPC SUVs were quite similar, as presented in **Figure 2.4A,B** [84]. Chungcharoenwattana and colleagues confirmed the

matrix effect by employing a particular system, gel filtration chromatography (also known as size exclusion chromatography) combining with DLS, to measure the detailed size distribution of newly formed phospholipid/oleate liposomes across individual elution fractions [83]. Using freeze-fracture electron microscopy, it was also explored how adding different amounts of oleate to preexisting phospholipid liposomes affects the resulting liposome size distribution, whereby the addition of a small amount caused a narrower size distribution of newly formed mixed liposomes than the addition of a large amount, as shown in **Figure 2.4C,D** [87]. At a mechanistic level, it has been suggested that fatty acid interactions cause membrane fission and partial solubilization, giving rise to the matrix effect and related phenomena [87, 88]. The effects of adding various fatty acids to preexisting DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) or POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) SUVs have also been determined by turbidity measurements based on UV/VIS spectrophotometry experiments [85]. The fatty

Figure 2.4 DLS measurements and complementary freeze-fracture electron micrographs measuring the interaction of fatty acids with SUVs and LUVs. DLS-measured size distribution curves of POPC lipid vesicle extruded with (A) 50 nm, (B) 100 nm diameter pore filters before (curve b) and after (curve c) oleate addition [84], and freeze-fracture micrographs of (C) preformed 180-nm diameter Egg PC vesicles and (D) Egg PC/oleate (1:1) vesicles. The scale bars are 200 nm [87].

acids exhibited rapid incorporation into the preexisting liposomes, followed by the formation of mixed phospholipids/fatty acid liposomes through size growth and subsequent fission. These changes were determined by noticing that the turbidity increased dramatically in the presence of preexisting liposomes as compared when preexisting liposomes were absent. Hence, solution-phase liposomes provide a useful platform for investigating the types of morphological changes that occur when antimicrobial lipids are added to phospholipid membranes. As remarked above, only a single fatty acid molecule has been studied using this approach and no monoglycerides have been studied. It should be emphasized that the tested molecule is not a classical antimicrobial lipid, and is a more generally studied fatty acid. The existing studies in the field have not been conducted within the purview of antimicrobial lipids. Furthermore, it is difficult to correlate the molecular self-assembly of antimicrobial lipids (or more generally lipid molecules) in the bulk solution with the measured effects because the interactions are highly dependent on the ratio of phospholipids to antimicrobial lipid.

Table 2.5 Investigation of fatty acid interactions with solution-phase liposomes.

Liposome (Composition)	Fatty Acid /Anion	Techniques	Key Observations	Ref.
SUVs (POPC)	Oleate	Electron microscopy Dynamic light scattering UV/VIS spectrophotometry	Different molar ratio of oleate were added to preexisting POPC liposomes, and caused varying changes in the size and number of newly formed mixed liposomes. At a low molar ratio of oleate to POPC liposomes, the size of resulting mixed liposomes remained similar, while at high ratios, the mixed liposomes were larger and became polydisperse.	[82]
SUVs (POPC)	Oleate	Cryo-TEM UV/VIS spectrophotometry	Oleate addition to preexisting POPC liposomes induced rapid formation of POPC/oleate mixed liposomes with increased diameter and total number. Only a few smaller mixed liposomes were generated by induced fission processes.	[89]

Table 2.5 *Cont.*

Liposome (Composition)	Fatty Acid /Anion	Techniques	Key Observations	Ref.
SUVs (POPC)	Oleate	Cryo-TEM	Equimolar addition of oleate to preexisting POPC liposomes led to smaller size distributions of new mixed liposomes, and occurred <i>via</i> subsequent fission processes.	[86]
SUVs (POPC)	Oleate	Dynamic light scattering Optical density	Addition of oleate to preexisting POPC SUVs (50, 100 nm) induced formation of mixed POPC/oleate liposomes with similar size distributions to original ones.	[84]
SUVs, LUVs (Egg PC)	Oleate	Gel filtration chromatography combined with dynamic light scattering (DLS) UV/VIS spectrophotometry	In the presence of Egg PC vesicles, oleate induced rapid spontaneous vesiculation, forming new EggPC/oleate vesicles. Size distribution of the formed EggPC/oleate vesicles depended on preexisting Egg PC vesicle size.	[83]
SUVs, LUVs (Egg PC)	Oleate	Electron microscopy Dynamic light scattering Gel exclusion chromatography	Size distribution of mixed phospholipid/oleate liposomes depended on the amount of oleate added to preexisting phospholipid liposomes. Small amount of added oleate induced a narrower size distribution of the mixed liposomes, as compared to when a larger amount was added.	[87]
SUVs, LUVs (Egg PC)	Oleate	Gel exclusion chromatography	Addition of oleate to preexisting liposomes induced the formation of smaller mixed phospholipid/oleate liposomes through two different mechanisms, fission and partial solubilization.	[88]
SUVs (DMPC, POPC)	Capric acid, Oleic acid, Linoleic acid	Electron microscopy Dynamic light scattering Light microscopy UV/VIS spectrophotometry	Rapid incorporation of fatty acids into preexisting liposomes induced formation of mixed phospholipid/fatty acid liposomes through size growth and subsequent fission. Fatty acids were more likely to incorporate into the preexisting liposomes than forming fatty acid vesicles themselves.	[85]

2.2.2.2 Giant Unilamellar Vesicle

To directly visualize membrane morphological changes induced by antimicrobial lipids, giant unilamellar vesicles (GUVs) in solution-based systems have been utilized to monitor interaction kinetics. Typically, the GUVs have greater than 10 μm diameter and are hence able to be studied by optical microscopy and fluorescence microscopy using fluorescent-labeled phospholipids for visualizing phenomena such as phase separation [90-99]. While SUVs and LUVs are typically studied by ensemble-average techniques, individual GUVs can be directly studied and a wide amount of information about morphological behavior, including fluctuations and membrane fission/fusion, can be monitored in real-time. By performing measurements on a large number of individual GUVs, the data can be processed through statistical methods to provide deep understanding of membrane interaction processes [90, 93]. Such approaches have led to a wide range of GUV studies to investigate various classes of membrane-interacting agents, including peptides [90, 91], flavonoids [93], and lanthanides [92]. In general, phase-contrast and fluorescence microscopy have been used to monitor GUV responses.

Of note, after treating GUVs with representative membrane-active, nonionic surfactants, the morphological responses have been observed in real-time, and Tamba *et al.* observed membrane disruption and leakage in response to treatment with Triton X-100 and octylglucoside [94]. Mavčič *et al.* assessed the influence of another nonionic surfactant, octaethyleneglycol dodecylether (C_{12}E_8), on GUVs, and dynamic morphological responses such as the shape transformation from tubular to spherical formations depending on the C_{12}E_8 concentration [95]. Of particular relevance to this thesis, the interaction of various single-chain lipid amphiphiles with GUVs has also been investigated. Inaoka *et al.* investigated membrane fission in GUVs that occurred upon treatment of lysophosphatidylcholine (LPC) containing 16-carbon long alkyl chains at low concentration, *e.g.*, 2 μM LPC, along with varying the membrane composition and amphiphile concentrations [96]. Tanaka *et al.* also observed similar membrane fission behavior with interesting shape changes occurring as well. In the study, LPC molecules with different chain lengths ranging from 10 to 16 carbons long were tested against 40 mol% cholesterol-containing GUVs, and the resulting shape changes varied from prolate

to asymmetrical spherical shape and membrane fission occurred above a corresponding threshold concentration for each LPC (which decreased with increasing chain length) [97]. Furthermore, Peterlin *et al.* monitored morphological responses of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) GUVs upon treatment with 0.8 mM oleic acid solution, under which condition exists in the mixed oleic acid/oleate liposome form [98]. Upon treatment, the GUVs started growing followed by various responses such as membrane invaginations, evaginations and budding, and finally creating small budding liposomes that were attached to the mother GUV, as presented at **Figure 2.5**. Recently, Mally *et al.* utilized phase-contrast microscopy to characterize the interactions between oleic acid and GUVs, revealing how fatty acid insertion causes an increase in GUV size followed by bursting [99]. A physical model was developed to explain the results, with

Figure 2.5 Optical micrographs showing the morphological responses of POPC GUVs that occur upon treatment with 0.8 mM oleic acid solution [98].

particular focus on the role of membrane strain in triggering the burst after reaching a critical threshold. As such, at an observational level, GUV platforms have provided a useful tool to study the interactions between antimicrobial lipids and phospholipid membranes. As with other liposome-based systems, only fatty acids have been studied, and not monoglycerides, and the study focus has been on a broader set of questions rather than focusing on antibacterial activities.

2.2.2.3 Supported Lipid Bilayers

Expanding beyond solution-phase studies, the supported lipid bilayer (SLB) platform has emerged as a promising measurement platform to study the mechanism of action of fatty acids and monoglycerides interacting with phospholipid membranes. SLB platforms are composed of two-dimensional phospholipid bilayers that are supported on a hydrophilic support and the bilayer-substrate interaction stabilizes the model membrane [100] while preserving key functional features of membranes in general. One particular advantage of SLB platforms is that they can be studied by a wide range of surface-sensitive measurement techniques, thereby allowing detailed investigation of the interaction kinetics from multiple perspectives, including binding mass, change in viscoelastic properties, and membrane fluidity.

As mentioned above, the insertion of antimicrobial lipids and other single-chain lipid amphiphiles into phospholipid bilayers induces membrane strain, and the bilayers can undergo membrane remodeling in order to respond to the applied strain. Staykova *et al.* observed how osmotic pressure changes can generate membrane strain in SLB platforms and presented a physical model to describe the resulting remodeling processes [101]. Specifically, the SLB platform responds by deforming to form spherical or tubular shapes protruding from the bilayer. With increasing compressive strain, the morphological response shifted from spherical to tubular protrusions, as monitored by confocal microscopy. In addition, Cambrea *et al.* showed that spherical protrusions can form on fluid-phase SLBs composed of phosphatidylcholine and phosphatidic acid lipids in response to changing ionic strength conditions, as determined by fluorescence microscopy [102]. The presence of phosphatidic acid molecules in the SLB caused a

large negative spontaneous curvature in the bilayer, leading to deformation of the membrane to induce spherical caps when the SLB were exposed to asymmetric osmotic pressure conditions. It was identified that different geometries of the intercalating lipid molecules significantly affected membrane strain. Following this line, Seu *et al.* reported detailed characterization of how single-chain lipid amphiphiles affect SLB properties by correlating changes in membrane fluidity with corresponding lipid-phospholipid intermolecular interactions on the basis of fluorescence recovery after photobleaching (FRAP) and attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) experiments [103]. Specifically, it was determined that insertion of a single-chain lipid amphiphile, lysophosphatidylcholine (LPC), in the SLB platform increased membrane fluidity by reducing the interactions between phospholipid molecules.

Following this line, several studies have been conducted reporting the direct observation of membrane morphological responses upon treating preformed SLB platforms with single-chain lipid amphiphiles, including fatty acids and monoglyceride, as summarized at **Table 2.6**. Giger *et al.* treated SLB platforms composed of dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidic acid (DOPA) phospholipids with C16 LPC, and monitored resulting membrane responses by time-lapsed fluorescence microscopy and FRAP. As presented in **Figure 2.6A,B**, elongated tubule structures were observed at or above 50 μM LPC, and a subsequent decrease in the ionic strength conditions transformed the lipid structures from tubule to spherical caps with complex morphologies [100].

Within the specific context of fatty acids, Thid *et al.* observed similar tubule formation resulting from adding docosahexaenoic acid (DHA), a polyunsaturated long fatty acid having $\text{pK}_a \sim 7.7$, to POPC SLBs [104]. This study was particularly important because it demonstrated the combined use of the quartz crystal microbalance-dissipation (QCM-D) and fluorescence microscopy techniques to study membrane morphological responses in complementary fashion. Moreover, this study was significant because it was the first investigation reporting the interaction of a fatty acid molecule with an SLB platform and provided initial evidence suggesting that DHA causes tubule formation above its corresponding CMC value. The fluorescence microscopy results provided direct evidence of the tubule formation, as evidenced in **Figure 2.6C,D**, while the QCM-D

Figure 2.6 Fluorescence micrographs depicting the morphological responses of SLBs. The morphological responses occurred after treatment with 50 μM lysophosphatidylcholine (LPC) in (A) 250 mM KCl and (B) 50 mM KCl [100], and (C) 200 μM docosahexaenoic acid (DHA) and corresponding (D) proposed mechanism of tubule formation [104].

measurements provided corroborating data that DHA treatment increased the viscoelastic properties of the SLB platform. The combination of the two measurement techniques led the authors to conclude that the elongated tubules form in response to membrane strain arising from DHA incorporation. Recently, Flynn *et al.* conducted a more detailed QCM-D study investigating how DHA treatment affected SLBs composed of POPC alone, POPC and L- α -phosphatidylinositol (PI), or POPC and L- α -phosphatidylserine (PS), and monitored the corresponding interaction kinetics [105]. At or above 50 μM DHA, there was a large increase in adsorbed mass and dissipation on POPC SLBs, indicating significant strong interactions. However, with the introduction of negatively charged PS

or PI molecules to the SLB platform, the DHA interaction became attenuated, likely reflecting some degree of electrostatic repulsion between negatively charged SLBs and anionic DHA molecules. While the two aforementioned studies are the only ones reporting SLB platform studies involving fatty acids, Hyldgaard *et al.* also investigated how monicaprylate, the monoglyceride derivative of caprylic acid (C8:0), causes membrane destabilization on SLBs composed of *E. coli* bacterial lipid extracts. QCM-D measurements showed a monotonic increase in bound mass with increasing monicaprylate concentration from 0.05 mM upwards, and appreciable shifts were detected at 5 mM test concentration, however, it was difficult to interpret the origin of the measurement responses due to the complex compositional features of the SLB platform in this case. Using AFM, it was further shown that monicaprylate preferentially interacted with liquid-disordered phase regions of the SLB platform and caused membrane defects, suggesting that the membrane interaction of monicaprylate affected membrane fluidity [106]. In summary, DHA and monicaprylate are the only fatty acids and monoglyceride compounds that have been tested using SLB platforms. The studies summarized in this section support the potential of utilizing SLB platforms to study concentration-dependent interactions, although the potential for studying antimicrobial lipids remains largely untapped, and future opportunities are discussed in the following section.

Table 2.6 Interactions of antimicrobial lipids and related single-chain lipid amphiphiles with SLB platforms.

SLB Composition	Single-Chain Amphiphile	Techniques	Key Observations	Ref.
DOPC /PA	Lysophosphatidylcholine (LPC) (16:0 tail)	Fluorescence microscopy FRAP	At or above 50 μ M LPC concentration, elongated tubule protrusions formed from SLB. Decrease in ionic strength shifted membrane structure from tubule to spherical cap shape.	[100]
Egg PC	LPC Lysophosphatidylethanolamine (LPE)	FRAP ATR-FTIR	Addition of LPC increases bilayer fluidity, while addition of LPE decreased bilayer fluidity. Hydrogen bonding interactions between phosphate group of lipids and amine group (in PE headgroup) were cited as cause of decreased fluidity.	[103]
POPC	Docosahexaenoic acid (DHA)	QCM-D Fluorescence microscopy	Above CMC value of DHA (~ 60 μ M), DHA induce significant changes in the viscoelastic properties of the SLB platform. Treatment with 200 μ M DHA caused formation of elongated worm-like lipid (tubule) structures.	[104]
POPC, POPC/PS POPC/PI	Docosahexaenoic acid (DHA)	QCM-D	Effects on POPC SLB induced by DHA treatment were concentration-dependent and occurred at or above 50 μ M DHA. Incorporation of PS and PI into POPC SLBs decreased the interaction of DHA with the lipid bilayer.	[105]
Bacterial lipid extracts (<i>E. coli</i>)	Monocaprylate	QCM-D AFM	At or above 5 mM monocaprylate concentrations, significant changes in the viscoelastic properties of <i>E. coli</i> lipid SLBs occurred. It was suggested that monocaprylate interacts with SLBs in the liquid-disordered phase state and causes defect formation.	[106]

2.3 Outstanding Questions

2.3.1 Correlation between Biophysical and Biological Activities

To date, nearly all studies involving antimicrobial lipids have focused on assessing antibacterial activity against target bacteria. The principal goal of these studies was directed at characterizing the activity spectrum and potency of different antimicrobial lipids, including determining how chain length and degrees of unsaturation affect these biological activities. As described above, a wide range of methods and experimental conditions were tested, and the field largely consists of discrete empirical findings without achieving a broader understanding of why and how antimicrobial lipids work. It is currently understood that antimicrobial lipids principally target bacterial cell membranes, however, mechanistic distinctions between different antimicrobial lipids are lacking. Within this scope, a key question is what determines the relative potencies of two different antimicrobial lipids against a target bacterium? Does potency, or rather the concentration-dependent profile of antibacterial activity that an antimicrobial lipid exhibits, relate to the self-assembly of an antimicrobial lipid in bulk solution or the affinity between an antimicrobial lipid molecule(s) and a phospholipid membrane, or to another factor?

An additional complexity of broadly interpreting the existing studies is that the underlying experimental designs were largely influenced by the type of application that was foreseen for the particular bacterium under investigation. For example, when evaluating antimicrobial lipids against bacteria associated with food-borne illnesses, high concentrations of antimicrobial lipids were often used mimicking their possible role as preservatives in functional foods. On the other hand, when testing antimicrobial lipids against medically important bacteria, more attention was placed on determining minimum inhibitory concentrations. Hence, variations in the scope of antibacterial testing limit the generalizability of scientific findings, and emphasize the need to develop a more holistic experimental framework, preferably one that correlates the activity of antimicrobial lipids across the biophysical and biological levels.

Central to such questions is the need to characterize how antimicrobial lipids interact with phospholipid membranes, and to connect these insights with antibacterial activities. At present, the field is divided into the majority of studies focused on biological activities with concentration-dependent inhibitory reporting, and a minor group of studies focused on biophysical experiments (on a very limited subset of antimicrobial lipids that are almost always studied within the scope of different contexts) without drawing relevance to antibacterial testing. By devising experimental approaches that integrate biophysical and biological studies, it would be possible to determine how the molecular self-assembly of antimicrobial lipids influences concentration-dependent interactions with phospholipid membranes – mimicking the purported target of bacterial cells – and to correlate these insights with biological activities, including inhibitory activity against particular bacteria of interest.

2.3.2 Physicochemical Distinctions between Fatty Acids and Monoglycerides

In general, it is recognized that monoglycerides have greater antibacterial potency than free fatty acids of equivalent chain length. For example, the 12-carbon long GML is typically inhibitory at lower concentrations than LA. However, there is essentially no discussion about the physicochemical determinants behind this empirically observed trend. Simply put, why are monoglycerides more active than free fatty acids? From a chemical perspective, it is noted that monoglycerides are nonionic molecules with no net electrical charge under physiologically relevant pH conditions. In contrast, free fatty acids are ionizable molecules that possess a carboxylic acid functional group. Under roughly neutral pH conditions, the carboxylic acid will be deprotonated, rendering fatty acids anionic with a net electrical charge of -1. These distinct molecular properties suggest that fatty acids and monoglycerides have different physicochemical properties, and it remains to be determined if these differences are related to the variations in the potency of antibacterial activity.

To answer this question, it is necessary to employ biophysical approaches and, in turn, ask more fundamental questions comparing fatty acids and monoglycerides. In principle, both classes of antimicrobial lipids are single-chain lipid amphiphiles and, after

reaching a critical concentration, begin forming micelles in solution. This leads to the next question: how do the molecular properties of fatty acids and monoglycerides influence their respective CMC values? Furthermore, in corollary studies reporting how long-chain DHA fatty acid interacts with supported lipid bilayers, it was noticed that DHA is active only above its CMC value ($\sim 60 \mu\text{M}$, Ref. [107]). Following this line, do the antibacterial activities of fatty acids and monoglycerides – limited in scope to the medium-chain saturated compounds tested in this thesis – correlate with CMC values? Importantly, what can be learned by studying these compounds with supported lipid bilayers in a concentration-dependent fashion and do fatty acids and monoglycerides induce similar, or distinct, types of membrane morphological responses? Aside from effects of molecular properties on self-assembly behavior in solution, to what extent do the different molecular properties (*e.g.*, net electrical charge) of fatty acids and monoglycerides affect interactions with supported lipid bilayers? All of these questions form the basis for formulating the cross-disciplinary experimental strategy pursued in this thesis. The strategy represents a novel approach to characterize antimicrobial lipids by employing supported lipid bilayer platforms. It also facilitates the convergence of chemical and material science insights together with biological activity profiling. Taken together, these effects can guide the rational selection of testing conditions across biophysical and biological measurements.

References

- [1] H. Thormar, *Lipids and Essential Oils as Antimicrobial Agents*, John Wiley & Sons, (2010).
- [2] H. Thormar, H. Hilmarsson, *Chemistry and Physics of Lipids*, 150 (2007) 1-11.
- [3] Y.E. Chen, H. Tsao, *Journal of the American Academy of Dermatology*, 69 (2013) 143-155. e143.
- [4] T. Nakatsuji, R.L. Gallo, *Journal of Investigative Dermatology*, 132 (2012) 887-895.
- [5] R.I. Aminov, *Frontiers in Microbiology*, 1 (2010).
- [6] R. Laxminarayan, A. Duse, C. Wattal, A.K. Zaidi, H.F. Wertheim, N. Sumpradit, E. Vlieghe, G.L. Hara, I.M. Gould, H. Goossens, *The Lancet Infectious Diseases*, 13 (2013) 1057-1098.
- [7] J.J. Kabara, *Journal of the Society of Cosmetic Chemists*, 31 (1980) 1-10.
- [8] A.P. Desbois, V.J. Smith, *Applied Microbiology and Biotechnology*, 85 (2010) 1629-1642.
- [9] H. Kakisawa, F. Asari, T. Kusumi, T. Toma, T. Sakurai, T. Oohusa, Y. Hara, M. Chiharai, *Phytochemistry*, 27 (1988) 731-735.
- [10] L. Kumaratilake, B. Robinson, A. Ferrante, A. Poulos, *Journal of Clinical Investigation*, 89 (1992) 961.
- [11] E. Skřivanová, Z. Molatová, M. Marounek, *Veterinary Microbiology*, 126 (2008) 372-376.
- [12] A. P Desbois, *Recent Patents on Anti-Infective Drug Discovery*, 7 (2012) 111-122.
- [13] H. Maag, *Journal of the American Oil Chemists' Society*, 61 (1984) 259-267.
- [14] M. Barratt, *Toxicology in Vitro*, 10 (1996) 85-94.
- [15] P.H. Stahl, C.G. Wermuth, *Handbook of Pharmaceutical Salts: Properties, Selection, and Use*, John Wiley & Sons, (2008).
- [16] E.P. Serjeant, B. Dempsey, *Ionisation Constants of Organic Acids in Aqueous Solution*, Pergamon, (1979).
- [17] J.R. Kanicky, D.O. Shah, *Journal of Colloid and Interface Science*, 256 (2002) 201-207.

- [18] H. Osborn, C. Akoh, *Comprehensive Reviews in Food Science and Food Safety*, 1 (2002) 110-120.
- [19] R.P. Bazinet, S. Layé, *Nature Reviews Neuroscience*, 15 (2014).
- [20] J.J. Kabara, D.M. Swieczkowski, A.J. Conley, J.P. Truant, *Antimicrobial Agents and Chemotherapy*, 2 (1972) 23-28.
- [21] D. Greenway, K. Dyke, *Microbiology*, 115 (1979) 233-245.
- [22] M. Feldlaufer, D. Knox, W. Lusby, H. Shimanuki, *Apidologie*, 24 (1993) 95-99.
- [23] E. Kodicek, A. Worden, *Biochemical Journal*, 39 (1945) 78.
- [24] H. Galbraith, T. Miller, A. Paton, J. Thompson, *Journal of Applied Microbiology*, 34 (1971) 803-813.
- [25] H. Saito, H. Tomioka, T. Yoneyama, *Antimicrobial Agents and Chemotherapy*, 26 (1984) 164-169.
- [26] H.R. Knapp, M.A. Melly, *Journal of Infectious Diseases*, 154 (1986) 84-94.
- [27] J. Wille, A. Kydonieus, *Skin Pharmacology and Physiology*, 16 (2003) 176-187.
- [28] G. Bergsson, J. Arnfinnsson, Ó. Steingrímsson, H. Thormar, *Journal of Pathology, Microbiology and Immunology*, 109 (2001) 670-678.
- [29] C.Q. Sun, C.J. O'Connor, A.M. Roberton, *FEMS Immunology & Medical Microbiology*, 36 (2003) 9-17.
- [30] P. Heczko, R. Lütticken, W. Hryniewicz, M. Neugebauer, G. Pulverer, *Journal of Clinical Microbiology*, 9 (1979) 333-335.
- [31] H. Thormar, H. Hilmarsson, G. Bergsson, *Applied and Environmental Microbiology*, 72 (2006) 522-526.
- [32] J. Kabara, R. Vrable, M.L.K. Jie, *Lipids*, 12 (1977) 753-759.
- [33] L. Rohrer, K. Winterhalter, J. Eckert, P. Köhler, *Antimicrobial Agents and Chemotherapy*, 30 (1986) 254-257.
- [34] H. Thormar, C.E. Isaacs, H.R. Brown, M.R. Barshatzky, T. Pessolano, *Antimicrobial Agents and Chemotherapy*, 31 (1987) 27-31.
- [35] H. Hilmarsson, L. Larusson, H. Thormar, *Archives of Virology*, 151 (2006) 1217-1224.
- [36] J.J. Kabara, *Journal of the Society of Cosmetic Chemists*, 29 (1978) 733-741.
- [37] F. Kanetsuna, *Microbiology and Immunology*, 29 (1985) 127-141.

- [38] L.-L. Wang, E.A. Johnson, *Applied and Environmental Microbiology*, 58 (1992) 624-629.
- [39] B.W. Petschow, R.P. Batema, L.L. Ford, *Antimicrobial Agents and Chemotherapy*, 40 (1996) 302-306.
- [40] G. Bergsson, Ó. Steingrímsson, H. Thormar, *International Journal of Antimicrobial Agents*, 20 (2002) 258-262.
- [41] G. Bergsson, J. Arnfinnsson, S.M. Karlsson, Ó. Steingrímsson, H. Thormar, *Antimicrobial Agents and Chemotherapy*, 42 (1998) 2290-2294.
- [42] G. Bergsson, Ó. Steingrímsson, H. Thormar, *Antimicrobial Agents and Chemotherapy*, 43 (1999) 2790-2792.
- [43] M. Marounek, E. Skřivanová, V. Rada, *Folia Microbiologica*, 48 (2003) 731-735.
- [44] E. Skřivanová, O. Savka, M. Marounek, *Folia microbiologica*, 49 (2004) 199-202.
- [45] T. Kitahara, N. Koyama, J. Matsuda, Y. Aoyama, Y. Hirakata, S. Kamihira, S. Kohno, M. Nakashima, H. Sasaki, *Biological and Pharmaceutical Bulletin*, 27 (2004) 1321-1326.
- [46] T. Nakatsuji, M.C. Kao, J.-Y. Fang, C.C. Zouboulis, L. Zhang, R.L. Gallo, C.-M. Huang, *Journal of Investigative Dermatology*, 129 (2009) 2480-2488.
- [47] E. Skřivanová, M. Marounek, G. Dlouha, J. Kaňka, *Letters in Applied Microbiology*, 41 (2005) 77-81.
- [48] K. Benkendorff, A.R. Davis, C.N. Rogers, J.B. Bremner, *Journal of Experimental Marine Biology and Ecology*, 316 (2005) 29-44.
- [49] P.M. Schlievert, M.L. Peterson, *PloS one*, 7 (2012) e40350.
- [50] H. Kurihara, Y. Goto, M. Aida, M. Hosokawa, K. Takahashi, *Fisheries Science*, 65 (1999) 129-132.
- [51] S.-R. Won, M.-J. Hong, Y.-M. Kim, C.Y. Li, J.-W. Kim, H.-I. Rhee, *FEBS letters*, 581 (2007) 4999-5002.
- [52] N.R. Chamberlain, B. Mehrtens, Z. Xiong, F. Kapral, J. Boardman, J. Rearick, *Infection and Immunity*, 59 (1991) 4332-4337.
- [53] M. Salton, *Microbiology*, 5 (1951) 391-404.
- [54] P. Boyaval, C. Corre, C. Dupuis, E. Roussel, *Le Lait*, 75 (1995) 17-29.

- [55] S. Shin, V. Bajpai, H. Kim, S. Kang, *LWT-Food Science and Technology*, 40 (2007) 1515-1519.
- [56] D.P. Speert, L.W. Wannamaker, E.D. Gray, C.C. Clawson, *Infection and immunity*, 26 (1979) 1202-1210.
- [57] D.D. Carson, L. Daneo-Moore, *Journal of Bacteriology*, 141 (1980) 1122-1126.
- [58] L. Thompson, A. Cockayne, R. Spiller, *Gut*, 35 (1994) 1557-1561.
- [59] P.D. Boyer, *Annual Review of Biochemistry*, 66 (1997) 717-749.
- [60] P. Mitchell, *Nature*, 191 (1961) 144-148.
- [61] H. Galbraith, T. Miller, *Journal of Applied Microbiology*, 36 (1973) 659-675.
- [62] C.W. Sheu, E. Freese, *Journal of Bacteriology*, 111 (1972) 516-524.
- [63] J.S. Peters, C.-K. Chin, *Plant Physiology and Biochemistry*, 41 (2003) 117-124.
- [64] C.J. Zheng, J.-S. Yoo, T.-G. Lee, H.-Y. Cho, Y.-H. Kim, W.-G. Kim, *FEBS Letters*, 579 (2005) 5157-5162.
- [65] S.L. Sado-Kamdem, L. Vannini, M.E. Guerzoni, *International Journal of Food Microbiology*, 129 (2009) 288-294.
- [66] J.M. Andrews, *Journal of Antimicrobial Chemotherapy*, 48 (2001) 5-16.
- [67] I. Wiegand, K. Hilpert, R.E. Hancock, *Nature Protocols*, 3 (2008) 163-175.
- [68] H.M. Ericsson, J.C. Sherris, *Acta Pathologica et Microbiologica Scandinavica*, (1971).
- [69] B.K. Yoon, J.A. Jackman, M.C. Kim, N.-J. Cho, *Langmuir*, 31 (2015) 10223-10232.
- [70] I. Shibasaki, N. Kato, *The Pharmacological Effect of Lipids*, 1978 (1978) 15-24.
- [71] D. Dervichian, *Progress in the Chemistry of Fats and Other Lipids*, 2 (1954) 193-242.
- [72] H. Galbraith, T. Miller, *Journal of Applied Microbiology*, 36 (1973) 647-658.
- [73] R.D. Miller, K.E. Brown, S.A. Morse, *Infection and Immunity*, 17 (1977) 303-312.
- [74] C.A. Arias, B.E. Murray, *New England Journal of Medicine*, 360 (2009) 439-443.
- [75] G. French, *Journal of Antimicrobial Chemotherapy*, 58 (2006) 1107-1117.
- [76] M.M. Freundlich, *Science*, 142 (1963) 185-188.
- [77] E. Ruska, *Reviews of Modern Physics*, 59 (1987) 627.
- [78] D.B. Williams, C.B. Carter, *The Transmission Electron Microscope*, Springer, (1996) 3-17.
- [79] N.E. Gabriel, M.F. Roberts, *Biochemistry*, 23 (1984) 4011-4015.

- [80] M. Angelova, S. Soléau, P. Méléard, F. Faucon, P. Bothorel, In Preparation of Giant Vesicles by External AC Electric fields. Kinetics and Applications, Springer, (1992) 127-131.
- [81] E. Blöchliger, M. Blocher, P. Walde, P.L. Luisi, The Journal of Physical Chemistry B, 102 (1998) 10383-10390.
- [82] S. Lonchin, P.L. Luisi, P. Walde, B.H. Robinson, The Journal of Physical Chemistry B, 103 (1999) 10910-10916.
- [83] S. Chungcharoenwattana, M. Ueno, Chemical and Pharmaceutical Bulletin, 52 (2004) 1058-1062.
- [84] S. Rasi, F. Mavelli, P.L. Luisi, The Journal of Physical Chemistry B, 107 (2003) 14068-14076.
- [85] M.L. Rogerson, B.H. Robinson, S. Bucak, P. Walde, Colloids and Surfaces B: Biointerfaces, 48 (2006) 24-34.
- [86] N. Berclaz, E. Blöchliger, M. Müller, P.L. Luisi, The Journal of Physical Chemistry B, 105 (2001) 1065-1071.
- [87] S. Chungcharoenwattana, H. Kashiwagi, M. Ueno, Colloid and Polymer Science, 283 (2005) 1180-1189.
- [88] S. Chungcharoenwattana, M. Ueno, Chemical and Pharmaceutical Bulletin, 53 (2005) 260-262.
- [89] N. Berclaz, M. Müller, P. Walde, P.L. Luisi, The Journal of Physical Chemistry B, 105 (2001) 1056-1064.
- [90] Y. Tamba, M. Yamazaki, Biochemistry, 44 (2005) 15823-15833.
- [91] Y. Yamashita, S.M. Masum, T. Tanaka, M. Yamazaki, Langmuir, 18 (2002) 9638-9641.
- [92] T. Tanaka, Y. Tamba, S.M. Masum, Y. Yamashita, M. Yamazaki, Biochimica et Biophysica Acta (BBA)-Biomembranes, 1564 (2002) 173-182.
- [93] Y. Tamba, S. Ohba, M. Kubota, H. Yoshioka, H. Yoshioka, M. Yamazaki, Biophysical Journal, 92 (2007) 3178-3194.
- [94] Y. Tamba, T. Tanaka, T. Yahagi, Y. Yamashita, M. Yamazaki, Biochimica et Biophysica Acta (BBA)-Biomembranes, 1667 (2004) 1-6.

- [95] B. Mavčič, B. Babnik, A. Iglič, M. Kandušer, T. Slivnik, V. Kralj-Iglič, *Bioelectrochemistry*, 63 (2004) 183-187.
- [96] Y. Inaoka, M. Yamazaki, *Langmuir*, 23 (2007) 720-728.
- [97] T. Tanaka, R. Sano, Y. Yamashita, M. Yamazaki, *Langmuir*, 20 (2004) 9526-9534.
- [98] P. Peterlin, V. Arrigler, K. Kogej, S. Svetina, P. Walde, *Chemistry and Physics of Lipids*, 159 (2009) 67-76.
- [99] M. Mally, P. Peterlin, S.a. Svetina, *The Journal of Physical Chemistry B*, 117 (2013) 12086-12094.
- [100] K. Giger, E.R. Lamberson, J.S. Hovis, *Langmuir*, 25 (2008) 71-74.
- [101] M. Staykova, M. Arroyo, M. Rahimi, H.A. Stone, *Physical Review Letters*, 110 (2013) 028101.
- [102] L.R. Cambrea, J.S. Hovis, *Biophysical Journal*, 92 (2007) 3587-3594.
- [103] K.J. Seu, L.R. Cambrea, R.M. Everly, J.S. Hovis, *Biophysical Journal*, 91 (2006) 3727-3735.
- [104] D. Thid, J.J. Benkoski, S. Svedhem, B. Kasemo, J. Gold, *Langmuir*, 23 (2007) 5878-5881.
- [105] K.R. Flynn, L.L. Martin, M.L. Ackland, A.A. Torriero, *Langmuir*, 32 (2016) 11717-11727.
- [106] M. Hyldgaard, D.S. Sutherland, M. Sundh, T. Mygind, R.L. Meyer, *Applied and Environmental Microbiology*, 78 (2012) 2957-2965.
- [107] J. Serth, A. Lautwein, M. Frech, A. Wittinghofer, A. Pingoud, *The EMBO Journal*, 10 (1991) 1325-1330.

Chapter 3

Experimental Methodology

This chapter outlines the experimental strategies pursued in this project, including the integration of biophysical and biological approaches. Starting with description of the motivation behind the methodology and an overview of the tested materials, the rest of the chapter provides detailed description of the different experimental methods to study antimicrobial lipids, including self-assembly behavior in aqueous solutions, interactions with phospholipid membranes, and inhibitory activity against bacteria. Particular emphasis is placed on detailing the fabrication, characterization and application of supported lipid bilayer platforms to study membrane morphological responses induced by antimicrobial lipids with a wide range of surface-sensitive measurement techniques.

3.1 Rationale for the Selection of Materials and Experimental Techniques

In this chapter, the experimental methodology guiding the work in this thesis is explained in three main sections: (i) materials, (ii) materials synthesis, and (iii) experimental techniques. A key motivation behind the selection of experimental strategies pursued in this thesis is the integration of biophysical and biological approaches in order to draw connections between the antibacterial activities of fatty acids and monoglycerides and corresponding biophysical phenomena involving membrane morphological responses. In the past, most studies on antimicrobial lipids focused on indirect biological readouts that evaluated growth inhibition or cell death, or the direct effects of treatment on bacterial cell morphology. While such approaches enable detailed understanding of biological activities, it remains difficult to characterize underlying mechanistic features and improved strategies to monitor membrane interactions in real-time are needed. With growing interest to understand how antimicrobial lipids interact with phospholipid membranes, model membrane platforms demonstrate promising analytical capabilities to study morphological responses triggered by antimicrobial lipids. While the utilization of model membrane platforms to study antimicrobial lipids has been very limited (only one study involving one monoglyceride compound against SLBs with complex membrane compositions, and two other studies involving DHA fatty acid), it has been demonstrated that SLB platforms are sensitive to membrane interactions and can remodel in order to accommodate membrane strain caused by environmental and molecular factors. Hence, SLBs are attractive candidates as measurement platforms for characterizing antimicrobial lipids. To date, the related model membrane platforms have been used in discrete fashion and integrating them together with chemical and biological methods is a principal goal of this thesis towards better understanding the physicochemical determinants behind the activity of antimicrobial lipids, along with more systematic investigation of antimicrobial lipid compositions.

With respect to measuring biological activities, the main objective is to conduct antibacterial assays against selected bacterial strains. It is currently understood that the inhibitory activity of antimicrobial lipids against bacteria is linked to disruptive interactions with bacterial cell membranes, and establishing correlations between

biophysical and biological activities would provide insight into the underlying physicochemical behavior of antimicrobial lipids. In the materials section, and the chemical and biological reagents are described, including single-chain lipid amphiphiles such as fatty acids and corresponding monoglycerides, phospholipids and buffer reagents, and bacterial cell strains and antibacterial testing consumables. Among single-chain amphiphiles, particular focus is placed on medium-chain saturated fatty acids and monoglycerides, including the 12-carbon long fatty acid and monoglyceride pair, lauric acid and glycerol monolaurate, which have the highest demonstrated antibacterial activity against *S. aureus*. The 10-carbon long fatty acid and monoglyceride pair, capric acid and monocaprin, were also tested.

Secondly, description of materials synthesis is provided as pertains preparation of antimicrobial lipid solutions, supported lipid bilayer fabrication, and bacterial cell culture. Phospholipids and cholesterol, along with appropriate buffer reagents, were used to fabricate supported lipid bilayers. For antibacterial assays, quality control and methicillin-resistant strains of *S. aureus* were cultured with appropriate biological reagents. A detailed description of these methods is provided, with emphasis on experimental execution as well as the design rationale for the importance of key steps in the different processes.

Lastly, all experimental techniques and conceptual backgrounds are described in the final section. The molecular self-assembly of the tested antimicrobial lipids was tested by determining the critical micelle concentration (CMC) based on measuring the partition of a fluorescence probe by spectroscopic methods. While varying the bulk concentration of antimicrobial lipids according to the corresponding CMC values, quartz crystal microbalance-dissipation (QCM-D) measurements were conducted in order to monitor the concentration-dependent interactions with supported lipid bilayers. Since the QCM-D technique enables real-time monitoring of interaction kinetics, the results provide mechanistic insight into how the physicochemical properties of antimicrobial lipids influence morphological and mechanical responses. Conditions which led to membrane morphological responses were further characterized by time-lapsed fluorescence microscopy measurements, which provided direct evidence to complement the QCM-D measurements. The fluorescence recovery after photobleaching (FRAP)

technique was also employed to measure changes in membrane fluidity before and after treatment with test compounds. Finally, the minimum inhibitory concentrations (MICs) of antimicrobial lipids based on growth inhibition of *S. aureus* were determined under various environmental conditions, and provided the basis for correlating concentration-dependent biophysical and biological activities.

3.2 Materials

3.2.1 Single-Chain Lipid Amphiphiles

Lauric acid (LA), sodium dodecyl sulfate (SDS), and capric acid were obtained from Sigma-Aldrich (St Louis, MO). Glycerol monolaurate (GML) was purchased from Abcam (Cambridge, U.K.). Monocaprin was obtained from LGC Standards (Teddington, UK). All of the compounds except monocaprin were stored at room temperature, while monocaprin was stored at -20 °C per the manufacturer's instructions. Specific amounts of each compound were weighted out immediately before sample preparation. The chemical structures of the fatty acids, monoglycerides, and related compounds used in this thesis are presented in **Figure 3.1**. LA and GML have 12-carbon long saturated hydrocarbon chains, and SDS is a sulfated derivative of lauryl alcohol. On the other hand, capric acid and monocaprin have 10-carbon long saturated hydrocarbon chains.

Figure 3.1 Chemical structures of the tested antimicrobial lipids and related compounds used in this thesis.

All tested compounds have saturated hydrocarbon chains and therefore exhibit high chemical stability in ambient conditions, unlike compounds with unsaturated hydrocarbon chains that are prone to air oxidation.

3.2.2 Phospholipid Reagents

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Liss Rhod-PE fluorophore), cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). Zwitterionic DOPC lipids, with a gel-to-fluid phase transition temperature of -17 °C, were used to form single-component SLB platforms at room temperature. Cholesterol was used for preparing cholesterol-containing SLB platforms as described below. Liss Rhod-PE fluorophore is a synthetic, negatively charged lipid that is a derivative of the DOPC phospholipid that possesses a fluorescently labelled head group, with excitation and emission wavelengths of 560 nm and 583 nm, respectively. All phospholipids were stored at -20 °C in either powder or chloroform form, and stock solutions were freshly prepared immediately before experiments.

Figure 3.2 Chemical structures of the tested phospholipids and cholesterol in this thesis.

3.2.3 Buffer Reagents

Two different buffer agents were used in the experimental studies. To promote SLB formation, a Tris buffer solution [10 mM Tris, 150 mM NaCl, pH 7.5] [1] was used as the running buffer for QCM-D and fluorescence microscopy experiments. After SLB fabrication was completed, the buffer solution was exchanged to phosphate-buffered saline (PBS) solution [pH 7.2], which was purchased from Gibco (Carlsbad, CA). The Tris buffer was selected for SLB fabrication because it has been successfully used in validation of the fabrication protocol and is popular for biotechnological experiments with high buffer capacity, with a pK_a value of 8.06 at 25 °C and an effective buffering range around pH 7.5 to 9 [2, 3]. In previous studies, SLBs were successfully fabricated on silicon dioxide surfaces with Tris buffer solutions by using the solvent-assisted lipid bilayer (SALB) method, which was chosen for the work in this thesis [4-6]. Also, pilot studies conducted during the initial stages of this thesis work indicated that SLB fabrication was more successful when using Tris buffer solution than PBS solution. However, the buffering capacity of Tris buffer is relatively low for the physiological pH range around 7.0 and 7.5 [1], and PBS solution was utilized for the biophysical and biological experiments in this thesis. The PBS buffer [pH 7.2] consists of 1.54 mM KH_2PO_4 , 155.17 mM NaCl, and 2.71 mM $Na_2HPO_4 \cdot 7H_2O$, and is a representative buffer for mimicking biological conditions [7]. For some experiments, phosphate-buffered water without additional salts was prepared. As appropriate, all Tris, PB, and PBS buffers were prepared in Milli-Q-treated water ($>18 M\Omega \cdot cm$) (Millipore, Billerica, MA).

3.2.4 Bacterial Cell Strains and Antibacterial Testing Reagents

Two *Staphylococcus aureus* strains, the quality control strain labeled as ATCC 25923 (Ref. [8]) and a methicillin-resistant strain labeled as ATCC 33591, were obtained from American Type Culture Collection (Manassas, VA). The ATCC 25923 strain has been used as a popular quality control stain in laboratories since 1945, and the ATCC 33591 strain is a pathogenic version that can cause serious skin infections. It is known that *S. aureus* is susceptible to treatment with LA and GML, and hence it provided a

suitable bacterium for correlating concentration-dependent biophysical and biological activities in this thesis. The bacterium was supplied in freeze-dried condition, and the cells were propagated by suspending them in Luria-Bertani (LB) broth (BD, Sparks, MD) which consists of tryptone (10 g/l), yeast extract (5 g/l), sodium chloride (10 g/l). After propagation, the bacterial cells were suspended in a 50 % v/v glycerol stock solution and stored at -80 °C until further use. LB broth was used for all steps of bacterial cell propagation and culture, and was the medium for optical density measurements at 600 nm wavelength (OD_{600}) [9]. Mueller-Hinton (MH) broth, which is made of beef infusion solids (2 g/l), starch (1.5 g/l), and casein hydrolysate (17.5 g/l), and agar (both from Sigma-Aldrich, St Louis, MO) were used in the minimum inhibitory concentration (MIC) assay experiments. In Chapter 4, the antimicrobial lipid solutions for the MIC assay were prepared in PB, PBS, or MH broth depending on the particular experiment. In Chapter 7, the antimicrobial lipid solutions were exclusively prepared in PBS. All buffer and culture media for bacterial cell culture and antibacterial testing were sterilized using an autoclave at 121 °C for 15 mins and stored at 4 °C in sterile conditions until experiment.

3.3 Materials Synthesis

3.3.1 Preparation of Antimicrobial Lipid Solutions

Due to the poor solubility of anionic fatty acids in aqueous solution [10], some studies have reported dissolving fatty acids in 5 % v/v dimethylsulfoxide (DMSO) for preparing initial stock solutions [11-13]. Since DMSO is a polar aprotic solvent that can cause skin irritation [14], an alternative route for preparing stock solutions was used in this thesis, whereby antimicrobial lipids were initially dissolved in ethanol [15]. The appropriate mass of fatty acid or monoglyceride powder was weighed by using an analytical balance, and the powder sample was dissolved in absolute ethanol to prepare the stock solution. Typically, the ethanol stocks were diluted 100-fold in PBS. In Chapters 4 and 6, LA and GML were prepared in ethanol to obtain 200 mM stock solutions followed by dilution to 2 mM in PBS (1% ethanol then further diluted for experiments). The water-soluble surfactant, SDS, was directly prepared with PBS

(containing 1% ethanol) to achieve a 2 mM stock solution. In Chapter 5, capric acid and monocaprin were prepared in ethanol to obtain 400 mM stock solution and then diluted to 4 mM in PBS 1% ethanol then further diluted for experiments. The 2-4 mM stock solutions in PBS were heated in a water bath at 70 °C for 30 mins in order to promote solubilization, and the stock solutions were extensively vortexed immediately before and after heating. After heating and vortexing was finished, the stock solutions were cooled down to room temperature, diluted to the appropriate concentration (typically in two-fold dilution series), and applied to the experiment. As mentioned above, some of the antibacterial tests were conducted in PB or MH broth conditions and the antimicrobial lipids used in these experiments were prepared according to the protocol described above, with PB or MH broth replacing PBS. In all cases, the sample solutions were freshly prepared immediately before experiment.

Regarding the preparation of LA and GML mixtures as described in Chapter 7, the appropriate mass of LA and GML were independently weighted and dissolved in absolute ethanol, yielding 200 mM ethanol stocks of each compound. The LA and GML ethanol stock solutions were each diluted in PBS to obtain the desired molar ratio. Separate PBS stock solutions were then prepared for LA and GML, respectively, and heated at 70 °C for 30 mins. Then, equal volumes of the stock solutions were mixed immediately after heating, followed by extensive vortexing of the mixture. The stock solution of the mixture was prepared at 4 mM total concentration and further diluted immediately before experiment.

3.3.2 Supported Lipid Bilayer Preparation

Supported lipid bilayers (SLBs) were employed to investigate the membrane-activity of single-chain lipid amphiphiles. To date, some studies for monitoring fatty acid interactions with model membrane platforms have been conducted by using giant unilamellar vesicles (GUVs) in solution-based systems, and real-time monitoring led to observation of membrane invaginations, evaginations, and budding, along with an increase of membrane strain followed by membrane bursting in response of fatty acid interactions [16, 17]. At the same time, using planar SLB platforms is preferable to the

GUV system for several reasons such as high tolerance of asymmetric solution conditions [18] and two-dimensional phospholipid bilayer structure which is useful to study changes in morphological and mechanical properties of the membrane as probed by surface-sensitive measurement techniques [19]. Thus, two-dimensional planar lipid bilayers on solid supports are excellent membrane-mimicking platforms and the membrane composition can be varied to include different phospholipids and sterols.

Within this scope, a key goal in the field has been to develop simple and robust SLB fabrication that work with a wide range of membrane compositions. Over the years, various methods have been developed such as Langmuir–Blodgett (LB) deposition [20] and bubble collapse deposition [21]. Among conventional methods, liposome fusion is the most popular one and involves liposome adsorption and spontaneous rupture on solid supports (typically silica-based materials). Unilamellar liposomes adsorb onto solid supports and, under favorable conditions, the adsorbed vesicles can rupture spontaneously to induce formation of the SLB. Of direct relevance to this thesis, however, are the challenges associated with fabricating SLBs that contain high cholesterol fractions exceeding 30 mol% – a range which encompasses biologically relevant membrane compositions – when using the vesicle fusion method. In particular, surface-adsorbed vesicles containing high cholesterol fractions are unlikely to rupture due to high membrane bending rigidities [22]. Hence, SLBs can only be prepared with up to ~20 mol% cholesterol when the vesicle fusion method is used. The development of improved fabrication capabilities that bypass the requirements of liposome rupture would open the door to preparing SLBs with higher fractions of cholesterol.

Towards this goal, the recently reported solvent-assisted lipid bilayer (SALB) method has demonstrated promise as an alternative, and versatile, method to fabricate SLBs across a broader range of membrane compositions. Based on solvent-assisted lipid self-assembly, different bulk phase states of phospholipids in isopropanol/water mixture occur depending on the solvent fraction and creative use of these different phase states can lead to bottom-up SLB assembly. As presented in **Figure 3.3**, DMTAP/DMPC phospholipids exist in the inverted micelle form in isopropanol and, with increasing water fraction, the phospholipid assembly prefers the monomeric state, followed by normal micelles and lamellar-phase liposomes [23]. Inspired by these phase behaviors, Hohner *et*

al. established a method to deposit phospholipids in isopropanol (a water-miscible organic solvent) and then conduct a solvent-exchange step with aqueous buffer solution [23]. The gradual solvent-exchange leads to a series of phase transitions among deposited phospholipids on the solid support, and yields a lamellar-phase SLB with typical morphological and fluidic properties without requiring preparation or use of conventional liposomes.

Figure 3.3 Principle of solvent-assisted lipid self-assembly. Phospholipids are dissolved in pure isopropanol and exist as inverted micelles. A solvent-exchange step is conducted and the phase state of the phospholipids changes, yielding the bottom-up formation of an SLB on the solid support [23].

Inspired by solvent exchange self-assembly, a streamlined SALB protocol with rapid solvent-exchange was established, and it is able to form homogeneous SLBs on not only conventional silica-based surfaces but gold, titanium oxide, and aluminum oxide surfaces as well [4, 5]. Aside from fabricating phospholipid-based SLBs, another key advantage of the SALB method that is central to this thesis, is the capability to fabricate cholesterol-enriched SLBs with tunable amounts of cholesterol ranging between 0 and ~60 mol%, as presented at **Figure 3.4** [6]. In typical, experiments, the solid support is first incubated with aqueous buffer solution, followed by water-miscible organic solvent (usually isopropanol), then ~0.1 to 0.5 mg/ml phospholipid in the same water-miscible organic solvent, and finally a solvent-exchange step back to aqueous buffer solution. The experiments are conducted in a microfluidic flow-through chamber and the initial two steps are useful for quantifying the final SLB properties (relative to original baseline

values in aqueous buffer solution) and determining the amount of adsorbed lipid in the organic solvent. Depending on the membrane composition, some level of optimization is required and the protocol is also sensitive to the flow rate. For different membrane compositions reported in this thesis, rigorous experiments were done in order to independently optimize the SLB formation.

Figure 3.4 Fluorescence microscopy observation of cholesterol-rich SLBs with different cholesterol molar fractions, as prepared by the SALB method on glass surfaces [6].

3.3.3 Bacterial Cell Culture

Bacterial strains, namely a quality control strain of *S. aureus* and methicillin-resistant *S. aureus* (MRSA), were cultured in LB broth overnight at 37 °C. Following overnight culture, a certain amount of the bacterial cell culture was resuspended in fresh LB media with 4-5 v/v%, and then the bacterial suspension was grown at 37 °C until an OD₆₀₀ value of around 0.5 was reached, which corresponds to a cell density of $\sim 1 \times 10^8$ colony-forming units (CFU)/ml (exponential growth phase). The bacterial cells in exponential growth phase were harvested by centrifugation at 1500g for 10 mins. To wash the bacterial cells, the cell pellets were resuspended with the appropriate solutions (PB, PBS or broth medium depending on the particular experiment) and centrifuged again under the same condition. After two washing steps, the cells were resuspended in the appropriate medium, and the OD₆₀₀ value of the cell suspension was measured to obtain an OD₆₀₀ value of 0.1, which corresponds to a cell density of $\sim 2 \times 10^7$ CFU/ml, followed by consecutive dilution until reaching a final bacterial inoculum of around $5 \times$

10^5 CFU/ml. Freshly prepared cells were used in MIC assay experiments. For all experiments, 50 % v/v glycerol stock aliquots of each bacterium were made and stored at -80°C , and an aliquot was freshly used for overnight bacterial culture one day before an MIC assay experiment was conducted.

For CFU quantification, the bacterial cell culture that was prepared at an OD_{600} value of 0.1, was serially diluted in 10-fold increments with sterile PBS, and 10 μl from each diluent was streaked onto agar plates, followed by incubation for 24 h at 37°C . CFU/ml was determined by back-dilution. Based on routine verification experiments, it was established that an OD_{600} value of 0.1 corresponds to a cell density of $\sim 4 \times 10^7$ CFU/ml, such that a 40-times dilution yields a cell density of $\sim 1 \times 10^6$. **Figure 3.5** represents an example of CFU quantification for 20-times diluted sample of *S. aureus* cells that had an OD_{600} value of 0.1. Similar results for CFU quantification were obtained for the MRSA strain (ATCC 33591) as well.

Figure 3.5 Colony-forming unit (CFU) quantification result of *S. aureus* (ATCC 25923) cell culture at 20-times dilution for $\text{OD}_{600} = 0.1$. Agar plates containing between 25 and 250 cell colonies, which was the applicable range for plates with 10^3 dilution, were selected for CFU enumeration. A 20-fold diluted sample of *S. aureus* (ATCC 25923) cells with an original OD_{600} value of ~ 0.1 has a cell density of $\sim 2 \times 10^6$ CFU/ml. Noted that different streaking volumes of 10, 50, and 100 μl of diluent at each dilution factor were tested and based on similar results at all tested volumes, 10 μl was selected as the standard condition for all CFU quantification experiments.

3.4 Experimental Techniques

In this section, the measurement principles and method of analysis, along with detailed experimental protocol, are provided for experimental technique that was used in this thesis.

3.4.1 Critical Micelle Concentration Determination Assay

The critical micelle concentration (CMC) is defined as the concentration at which it becomes thermodynamically favorable for surface-active molecules to begin self-assembling into colloidal structures called micelles, and the CMC value represents an important property to characterize the aggregation behavior of different molecules. At sufficiently high bulk concentrations, surface-active amphipathic compounds such as antimicrobial lipids have a tendency to self-assemble into micelles in aqueous solution so that the hydrophobic tails of molecules do not come into contact with water molecules. Such behavior is referred to as the “hydrophobic effect”, and is based on reaching thermodynamically favorable states [24]. To determine the CMC values of fatty acids and monoglycerides in this thesis, the fluorescence probe technique was utilized and involves fluorescence spectroscopy experiments. There are several equally valid experimental approaches for CMC determination such as measuring surface tension or electrical conductivity method [25]. The fluorescence probe-based method was selected from among the options because the necessary equipment is readily available, and the approach is also applicable to non-ionic surfactants. Furthermore, the method has been validated through comparative surface tension measurements in previous works [26]. Briefly, fluorescence probe-based detection methods involve monitoring the spontaneous emission of light from an excited molecule that absorbed light. Typically, the emitted light has a longer wavelength with corresponding lower energy than that of the absorbed light. The difference between the excitation and emission wavelengths is known as the “Stokes shift”, which is triggered by the electronic transition between different energy levels as described in **Figure 3.6**. Upon light absorption, ground-state electrons (S_0) are excited to a higher energy level (S_2). Excited electrons will then go down to a lower

energy level at the vibrationally excited state (S_1) by losing some energy through internal conversion and vibrational relaxation. Eventually, the electron will fall back to the ground state by through energy dissipation, resulting in light emission at a longer wavelength, which is termed fluorescence emission [27]. For the CMC experiments in this thesis, pyrenecarboxaldehyde, specifically 1-pyrenecarboxaldehyde, was selected as the florescent probe. The fundamental basis for detecting the onset of micelle formation is that the probe has different fluorescent emission properties in the presence and absence of micelles. Specifically, the peak wavelength of its spectrum decreases with increasing extent of micelle formation. In PBS buffer, the peak wavelength is 473 nm and it further decreases in the presence of micelles. In the experiments, the fluorescence emission spectrum of 1-pyrenecarboxaldehyde was measured by using a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Australia) in the presence of increasing concentrations of test compound.

Figure 3.6 Jablonski diagram depicting fluorescence excitation and emission steps [27].

As mentioned above, the fluorescence probe technique is widely used for determining the CMC of surface-active compounds, and generally involves fluorescent probes such as pyrene and pyrenecarboxaldehyde that have fluorescent properties derived from aromatic ring structures with hydrophobic properties. As such, the probes are sensitive to the polarity of the surrounding environment, and show different emission spectra depending on the exposure to micelles and other shifts in the local chemical environment. The shifts in fluorescence emission spectral properties, especially changes in the peak wavelength, can be used for determining the onset of micelle formation, *i.e.*,

defined as the CMC. Pyrene is typically used as the probe for this technique, however, its highly hydrophobic character limits its solubility, and utility, for measurements in fully aqueous systems. To address this issue, the introduction of an aldehyde group to the pyrene structure, pyrenecarboxaldehyde, improves molecular polarity and thereby increases solubility in aqueous solution (**Figure 3.7**).

Figure 3.7 Chemical structure of the fluorescence probes, pyrene and 1-pyrenecarboxaldehyde, respectively. 1-pyrenecarboxaldehyde was used to determine the CMC experiments in this thesis.

Of note, the pyrenecarboxaldehyde-based fluorescent probe method has been validated as a reliable method for CMC determination, and researchers have shown that measurement results obtained by this method agree with those obtained by the surface tension method [26]. It has been further shown that the fluorescence emission spectrum of pyrenecarboxaldehyde has a maximum-intensity wavelength (termed peak wavelength) that correlates with the solvent polarity (reflected by the dielectric constant of the solvent, or simply D) based on the following equation:

$$\lambda_{\max} = 0.52D + 431.5 \quad [\text{Eq. 3.1}]$$

In general, polar solvents have relatively high dielectric constants, *e.g.*, water has a D value around 80, while many aromatic and hydrocarbon solvents have values around 2 (*e.g.*, benzene and cyclohexane) [28]. The measured peak emission wavelength of 1-pyrenecarboxaldehyde was determined to be 472 nm [29] and 473 nm in water and PBS, respectively, as presented in **Figure 3.8**. Based on Eq. 3.1, these results correspond to D values of ~78 and ~80 for deionized water and PBS solution, respectively. These values agree with the range of literature values as well [30].

Figure 3.8 Fluorescence spectrum of 1-pyrenecarboxaldehyde in PBS solution upon excitation at 365.5 nm wavelength.

When incubated with test compounds in the monomeric state in PBS solution, the peak wavelength of the fluorescence emission spectrum of 1-pyrenecarboxaldehyde is consistently similar to the control values (in the absence of test compound), with a value around 473 nm. The peak wavelength starts to decrease when micelles begin forming, indicating that the probe is intercalating into the hydrophobic micellar environment and thereby decreasing the local solvent polarity. This leads to a decrease in the dielectric constant of the local environment, and in turn the peak wavelength decreases accordingly. Thus, the highest concentration of test compound before a peak shift is observed is defined as the CMC. The resolution of the CMC determination depends on the concentrations of test compound that are tested, and in this thesis, pilot experiments were typically conducted across a broad concentration range before conducting a more narrow investigation within a specific concentration range. This combination facilitated efficiency and accuracy. As presented in **Figure 3.9**, a reference surfactant, SDS, with a known CMC value, was first evaluated to validate the measurement approach, and CMC values of 7 mM and 800 μ M were obtained in water and PBS, respectively. The recorded CMC value in water agrees well with literature values [25, 31, 32].

Figure 3.9 Determination of critical micelle concentration using the 1-pyrenecarboxaldehyde fluorescent probe. Peak wavelength is presented as a function of surfactant concentration in (A) water and (B) PBS. The PBS buffer [pH 7.2] consists of 1.54 mM KH_2PO_4 , 155.17 mM NaCl, and 2.71 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$.

Of note, the CMC values reported in the thesis have been summarized in **Table A.2**. For Chapter 4, the CMC values of test compounds (SDS, LA, and GML) were obtained from literature references in similar ionic strength conditions. For Chapter 5, the CMC values of capric acid and monocaprin were experimentally measured in equivalent solution conditions that matched those of the QCM-D and fluorescence microscopy experiments, and the protocol is described below.

Experimental Protocol:

First, a stock solution of 1-pyrenecarboxaldehyde was prepared by dissolving a certain mass of compound in methanol to obtain a final concentration of 5 mM (the 5 mM stock solution was stored at 4 °C and an aliquot was freshly diluted to 50 μM on the day of measurements). A certain amount of diluted probe was added to a glass vial and the sample was left in a fume hood for 30 mins to aid drying until the methanol evaporated completely. The result was a dry, thin film of the compound on the wall of the glass vial. An appropriate volume of PBS containing the test compound was used to hydrate the probe, until reaching a final probe concentration of 0.1 μM , followed by extensive vortexing. The 1-pyrenecarboxaldehyde molecules in the prepared samples were excited at 365.5 nm with a 10 nm slit width and the fluorescence emission spectra were scanned

from 400 nm to 600 nm. Each concentration of test compound was prepared in an individual glass vial that was coated with the probe and measured by using cuvette with a path length of 0.1 cm. All measurements were conducted for 6 scans at each test concentration and averaged. As presented in **Figure 3.10**, a representative emission spectrum shows that the peak shift decreases from 473 nm downward with increasing concentration of the test compound. The averaged peak wavelengths from 6 scans per concentration are plotted as a function of the test compound concentration. In this particular example, it is shown that the peak wavelength begins to decrease at 70 μM , and hence 60 μM is formally defined as the CMC value.

Figure 3.10 Representative CMC determination for the GML compound by the 1-pyrenecarboxaldehyde fluorescent probe method. (A) Fluorescence spectrum shift as a function of GML concentration and (B) Peak wavelength shift as a function of GML concentration in PBS solution.

3.4.2 Quartz Crystal Microbalance-Dissipation

Membrane interactions between antimicrobial lipids and supported lipid bilayers were investigated by employing the quartz crystal microbalance-dissipation (QCM-D) technique with the Q-Sense E4 instrument (Biolin Scientific, Stockholm, Sweden). The fundamental sensing principle of QCM-D measurements is that quartz crystal has piezoelectric properties, and is located between two electrodes across which an AC voltage is applied. Under the applied voltage, the quartz crystal mechanically oscillates

from periodic deformation with a characteristic resonance frequency that depends on the material properties of the quartz crystal. The typical resonance frequency of quartz crystal used in QCM-D is on the order of MHz, and the AT-cut quartz crystal sensor chips used in this thesis have a resonance frequency of 5 MHz [33-35]. Importantly, when additional mass adsorbs onto the sensor surface, there is a change in the resonance frequency, which can be related to the mass of adsorbed material on the sensor surface by the following equation (termed the Sauerbrey equation [36]):

$$\Delta m = \frac{C}{n} \times \Delta f \quad [\text{Eq. 3.2}]$$

where Δf is the frequency change, Δm is the adsorbed mass, n is the harmonic number and C is the sensitivity constant defined by:

$$C = \frac{t_q \times \rho_q}{f_o} \quad [\text{Eq. 3.3}]$$

where t_q is the thickness of the quartz crystal, ρ_q is the density of quartz, and f_o is the resonance frequency of the quartz crystal. The typical sensitivity constant for 5 MHz, AT-cut quartz crystal is around $-17.7 \text{ ng/cm}^2 \cdot \text{Hz}$ [37]. It is important to note that there are several conditions that must be fulfilled in order to apply the Sauerbrey equation. First, the amount of adsorbed mass on the sensor surface should be comparatively smaller than the crystal mass. Second, the adsorbed mass must be rigidly attached to the sensor surface and not slip from oscillatory motion. Third, the mass should be distributed homogeneously across the sensor surface. When the QCM-D technique was originally developed, it was intended for monitoring thin film deposition in vacuum environments and these conditions were typically satisfied. However, growing attention to liquid measurements have brought attention to the challenges of analyzing biomacromolecular adsorption data where adsorbates exhibit viscoelastic properties and are not rigidly attached to the sensor surface [38]. To address this issue, there have been intensive efforts to develop more sophisticated QCM-D measurement approaches based on simultaneously measuring changes in resonance frequency and energy dissipation, the latter of which is sensitive to the viscoelastic properties of the adsorbate. In particular, the energy

dissipation shift is assessed by monitoring the oscillation decay from turning off the driving voltage after exciting the quartz crystal at its resonance frequency (**Figure 3.11**).

Figure 3.11 Schematic representation of monitoring the oscillation decay in QCM-D measurements. (A) AC voltage is applied between electrodes. When the AC voltage is turned off, oscillation decay occurs and representative decay patterns for a (B) rigid and (C) viscoelastic adsorbate.

Based on the oscillation decay profile, the energy dissipation, D , is monitored and defined as follows [35]:

$$D = \frac{1}{\pi f \tau} \quad [\text{Eq. 3.4}]$$

where f is the frequency and τ is the decay time constant. It is a dimensionless value and related to the oscillation energy as follows [33]:

$$D = \frac{1}{Q} = \frac{E_{\text{dissipated}}}{2\pi E_{\text{stored}}} \quad [\text{Eq. 3.5}]$$

where Q is the quality factor, $E_{\text{dissipated}}$ is the dissipated energy during one cycle of oscillation, and E_{stored} is the stored energy during the oscillation. In short, D represents the sum of energy losses in the closed system.

Of particular relevance to this thesis, the QCM-D technique has proved particularly useful for characterizing model membrane platforms and can distinguish supported lipid bilayers from adsorbed vesicle layers on account of differences in hydrodynamically-coupled solvent (**Figure 3.12**). Research in this direction has been aided by the possibility to use oxide film-coated quartz crystal sensor chips whereby the quartz crystal acts as an indirect transducer and phospholipid adsorption onto the oxide film surface is tracked. Another important point about QCM-D measurements is that they

are sensitive to the amount of adsorbed biomolecules and hydrodynamically-coupled solvent and hence sensitive to morphological changes, as explored in this thesis. Depending on the application and specific features of the system under consideration, it is also possible to apply more complex models to analyze QCM-D measurement data, such as the Voigt-Voinova model [39].

Figure 3.12 Schematic representation of different model membrane platforms that are routinely studied in QCM-D applications, including adsorbed vesicle layer and supported lipid bilayer [40].

Experimental Protocol:

QCM-D experiments were conducted using a Q-Sense E4 instrument (Biolin Scientific, Stockholm, Sweden). The QCM-D sensor chips were sputter-coated with a 50-nm-thick layer of silicon dioxide, and had a fundamental frequency on 5 MHz (model no. QSX 303, Q-Sense AB). The experimental data were collected at the third ($n = 3$), fifth ($n = 5$), and seventh ($n = 7$) odd number overtones by utilizing the QSoft software program (Biolin Scientific). The data was normalized according to the overtone number, and data processing conducted with OriginPro 8.5 software (OriginLab, Northampton, MA). To

present data, the fifth ($n = 5$) odd overtone was used unless otherwise noted. Before experiment, the QCM-D sensor chips were rinsed with water and ethanol a few times and then dried by using nitrogen gas followed by treatment with oxygen plasma for 1 min with an Expanded Plasma Cleaner (model no. PDC-002, Harrick Plasma, Ithaca, NY). In order to fabricate single-component DOPC SLBs on the sensor surface with the SALB method, the measurements are stabilized in 10 mM Tris buffer (150 mM NaCl, pH 7.5). Following buffer stabilization, isopropanol was injected for 10 mins until equilibration and then 0.5 mg/ml DOPC lipid in isopropanol was added for 10 mins followed by exchange with Tris buffer solution (without lipid). The quality of fabricated SLBs was assessed by the final measurement value, with optimal values around -26 Hz for the frequency shift (Δf) and $<0.3 \times 10^6$ for the energy dissipation shift (ΔD).

In the case of fabricating cholesterol-enriched SLBs, the only modification from the protocol described above was that a 0.3 mg/ml mixture of DOPC and cholesterol at the desired molar ratio in isopropanol was added for 10 mins at the appropriate step. This concentration was selected based on optimal results from pilot experiments. Moreover, to confirm the amount of cholesterol in the fabricated SLBs, the bilayer was treated with 1 mM methyl- β -cyclodextrin (M β CD), which is able to extract cholesterol from the lipid bilayer. As presented in **Figure 3.13**, cholesterol depletion occurred after M β CD treatment of the bilayer, and the amount of cholesterol removed can be monitored by an increase in the Δf shift and analyzed to calculate the molar ratio of cholesterol in the SLB platform [6].

After SLB fabrication, the Tris buffer was exchanged to PBS [pH 7.2] in order to match up with biological test conditions. Afterwards, fatty acids or monoglycerides in PBS were injected and the interaction with SLBs and resulting membrane morphological responses were tracked in real-time by monitoring changes in mass and viscoelastic properties. All samples used during the SALB fabrication process were injected into the measurement chamber at a flow rate of 100 $\mu\text{L}/\text{min}$, and fatty acids or monoglycerides were injected at 50 $\mu\text{L}/\text{min}$ by using a peristaltic pump (Reglo Digital, Ismatec, Glattbrugg, Switzerland). The temperature in the measurement cells were maintained at 25.0 ± 0.5 °C during all QCM-D measurements.

Figure 3.13 Methyl-β-cyclodextrin treatment of cholesterol-containing SLB platforms. (A) Fluorescence micrograph of cholesterol-enriched SLB after MβCD treatment. (B) Increase amount of cholesterol depletion after 1 mM MβCD treatment as a function of cholesterol fraction used in the SLB fabrication [6]

3.4.3 Fluorescence Microscopy

Fluorescence microscopy is a popular measurement technique to observe biological specimens, including model membrane platforms [27]. It is particular type of optical microscope that is used for observing fluorescent materials with high selectivity. As mentioned when describing fluorescence spectroscopy in Section 3.4.1. above, fluorescence-based sensing techniques are centered around the basic principle of the “Stokes shift” [41]. When fluorophores in a sample are illuminated with light that covers the wavelength at which the fluorophore can absorb photon energy, the fluorophore emits light at a longer wavelength. The illuminated light can be filtered due to its weaker intensity, therefore only emitted light at the longer wavelength is detected by the emission filter and converted into an image. Epi-illumination fluorescence microscopy (termed epifluorescence microscopy) is widely used and utilizes light illumination from above the sample. **Figure 3.14** shows the basic components of an epifluorescence microscope. The full spectrum of light is sent from the arc lamp light source to the sample, through filter cube to emit light at a specific wavelength (green color) that

Figure 3.14 Schematic diagram of epifluorescence microscope setup [27].

illuminates the sample and excites fluorophores in the sample. The fluorophores are excited and emit light at a longer wavelength (red color), which can be detected. Beyond direct observation of biological cells [42-44], including bacterial cells [45], by utilizing fluorescence microscopy-based techniques, it has also been used extensively to characterize the properties of membrane and membrane-related components and to explore membrane protein [46] or peptide interactions [47] with SLBs. Of particular relevance to this thesis, the technique can be applied to directly observe membrane morphological responses induced by fatty acids and monoglycerides. Upon illumination of fluorophore-labeled phospholipids in the SLB (Liss Rhod-PE fluorophore was used in this thesis), the membrane morphological responses induced by test compounds can be directly observed in real time. **Figure 3.15** shows exemplary microscopic observations of membrane morphological changes upon treatment of SLBs with lysophosphatidylcholine (LPC) or docosahexaenoic acid (DHA). The brightly spotted tubule and small cap structures appear in marked contrast to the background, and protrude from the planar, as noted by their high fluorescence intensities. It should be noted that the SLB remains present in the background, but appears darker due to the difference in fluorescence intensity between the SLB and protruded structures, and corresponding effects of the two objects being in different focal planes, which also affect the observed intensities at a quantitative level [18, 48].

Figure 3.15 Microscopic observation of (A) LPC treatment on SLBs containing 30 mol% dioleoylphosphatidic acid (DOPA), 69.5 mol% DOPC, and 0.5 mol % tail-labeled nitrobenzofuran-conjugated PC (tail-labeled NBD-PC) [18] and **(B)** DHA treatment on SLBs composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphocholine (POPC) with additional 0.5 wt % of lissamine rhodamine B 1,2 dihexadecanoyl-*sn*-glycero-3-phosphaditylethanolamine (rhodamine-DHPE) [48].

Experimental Protocol:

Epifluorescence microscopy was conducted for direct observation of membrane morphological responses on SLBs, as triggered by fatty acids and monoglycerides. The experiments were conducted using an Eclipse TI-U inverted optical microscope (Nikon, Japan) with a 60 $\times$ magnification (NA = 1.49) oil-immersion objective lens (Nikon), and the images were collected with an iXon 512 pixel $\times$ 512 pixel EMCCD camera (Andor Technology, Northern Ireland). The pixel size was $0.267 \times 0.267 \mu\text{m}^2$. A fiber-coupled mercury lamp (Intensilight C-HGFIE, Nikon) was utilized to illuminate fluorophores with a TRITC filter set (excitation and emission wavelengths of 545 nm and 605 nm, respectively). First, the SLBs were fabricated by using the liposome fusion method, with the following lipid composition: 99.5 mol % DOPC lipid and 0.5 mol % 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) lipid. The liposomes were prepared with the standard Tris buffer [150 mM NaCl, pH 7.5] and

extruded through 50 nm-diameter pore, track-etched polycarbonate membranes (Whatman Schleicher & Schuell, Dassel, Germany), yielding liposomes with an average diameter size around 60 nm. The vesicle solution at 0.2 mg/ml was injected into microfluidic flow-through chamber (sticky slide VI 0.4, Ibidi, Germany) and then the SLBs formed from vesicle adsorption and spontaneous rupture on the glass surface enclosed within the chamber. After SLB fabrication, the buffer solution was exchanged from Tris to PBS buffer [pH 7.2], followed by injection of the test compound solution at a flow rate of 40 μ l/min. Upon compound treatment, time-lapsed micrographs were recorded every 5 s for a total duration of 30 min. The fluorescence intensity in the different micrographs was normalized as part of data processing.

3.4.4 Fluorescence Recovery After Photobleaching

The fluorescence recovery after photobleaching (FRAP) technique is widely used to investigate the fluidic properties of membrane components, including phospholipid molecules [49]. The FRAP technique is based on time-lapsed fluorescence microscopy-imaging of a fluorescently labelled SLB platform, which contains about 1-2 mol% of fluorescently labeled phospholipid that is mixed together with 98-99 mol% of unlabeled phospholipid. The fundamental principle behind FRAP measurements is that phospholipid molecules freely diffuse within the SLB platform and by illuminating a region of the SLB with a powerful laser, phospholipid molecules in that region can become “bleached” and lose fluorescent properties while retaining their molecular properties. Once the laser stops shining on that spot, the phospholipids in the bleached region will diffuse together with phospholipids in the rest of the SLB platform, and eventually the fluorescence intensity will become uniform again across the SLB platform. The rate of this fluorescence recovery is the parameter being measured and experimental protocols include three basic steps: measure fluorescence intensity of SLB before photobleaching, apply laser-induced photobleaching and then turn off, and finally monitor fluorescence recovery until reaching a maximum intensity. As presented in **Figure 3.16**, a region of interest (ROI) can be selected and the fluorescent properties of the SLB are typically detected by low-intensity light illumination. During the

photobleaching step, phospholipid molecules in the ROI are bleached quickly with a high-intensity light from a laser (typical power of 30-50 mW or greater). It is important to note that the bleached phospholipids are chemically identical, while the functional moieties are no longer fluorescent. During the post-bleaching stage (laser turned off and low-intensity illumination returns), the ROI initially appears in dark, and the bleached molecules start diffusing to unbleached regions while unbleached molecules diffuse to the bleached area. The time-dependent recovery of fluorescence intensity within the bleached region is tracked, and the recovery profile can be analyzed to determine the diffusion coefficient of phospholipid molecules as well as the mobile and immobile fractions [50].

Figure 3.16 Schematic representation of FRAP experiments conducted on an SLB platform.

(A) Uniform distribution of fluorescently labelled phospholipid molecules in the SLB, (B) Photobleaching of labeled phospholipid molecules in a particular region of the SLB, (C) Stop bleaching and labeled phospholipids begin to diffuse into the bleached spot, and (D) Recovery of fluorescence intensity in the bleached region and uniform distribution of labeled phospholipids across the SLB.

Briefly, the SLB can be treated as an isotropic system such that the recovery profile is equivalent across the substrate, and the recovery of fluorescently labelled molecules diffusing into the bleached area can be described by Fick's second law [51] as follows:

$$\frac{\partial c_{r,i}(r,t)}{\partial t} = D_i \nabla^2 c_{r,i}(r,t) \quad [\text{Eq. 3.6}]$$

where the rate of accumulation (denoted as the time derivative of the surface concentration of fluorescently labeled phospholipid, $c_{r,i}$) is proportional to the diffusion coefficient of the fluorescently labeled phospholipid such that t is the time (after bleaching), r is the radial distance from the center of the bleached area, and D_i is the diffusion coefficient of the i^{th} diffusing component. While FRAP measurements were originally designed for studying the fluidic behavior of biological membranes [52, 53], they are also very useful to study the fluidic properties of SLBs. To solve Eq. 3.6, there are several possible approaches and Jönsson *et al.* reported a particularly useful one called the Hankel transform method [54]. It has been experimentally validated and shown to produce accurate measurements of the diffusion coefficient even under non-ideal experimental conditions and is hence particularly robust. It was therefore used in this thesis to obtain the diffusion coefficient of SLB platforms, and it is important to note that FRAP measurements in this thesis were performed on fluid-phase SLBs whereby the fluorescently labeled probe had equivalent hydrocarbon chain properties to the predominant phospholipid and hence its diffusion coefficient provides a reasonable estimate of membrane fluidity in the system as a whole. Furthermore, information regarding the spatial distribution of fluorescently labelled probe molecules can be obtained when only one diffusion component exists in the system, which is valid for the experiments described in this thesis. Of particular relevance to this thesis, the FRAP technique was primarily used to confirm the quality of fabricated SLBs and also to monitor changes in the diffusion coefficient due to the interaction of antimicrobial lipids with phospholipid molecules in the SLB platform. The experimental results are presented in tabulated form in Chapter 5, while a representative example of the FRAP micrographs is presented in **Figure 3.17**. After a homogenous SLB platform was treated with 250 μM monocraprin, FRAP measurements were conducted and revealed that the diffusion

coefficient increased from $3.11 \pm 0.07 \mu\text{m}^2/\text{s}$ before treatment to $3.64 \pm 0.22 \mu\text{m}^2/\text{s}$ after treatment. Of note, the lateral diffusion coefficient of single-component DOPC SLB is typically around $2.2 \mu\text{m}^2/\text{s}$, while more complex compositions involving sterols can have lower mobility; for example, with 50 mol% cholesterol, DOPC-cholesterol SLBs have a diffusion coefficient of around $0.8 \mu\text{m}^2/\text{s}$ [6]. As such, there can be a wide range of lipid bilayer mobilities depending on the bilayer composition along with other factors such as lipid-substrate interaction strength [55] and surface roughness [56].

Figure 3.17 Representative time-lapsed FRAP micrograph snapshots depicting fluorescence recovery of a DOPC SLB. Before FRAP experiment, the SLB was treated with 250 μM monolaurin treatment. (A) SLB before photobleaching. (B) Time of photobleaching. (C) Recovery of fluorescence intensity in bleached over time.

Experimental Protocol:

Fabricated SLBs were exposed to a high-intensity light, and a 20 μm diameter circular spot was photobleached into the SLB by using a 532 nm, 100 mW laser (Klasech Laser Technologies, Dortmund, Germany). The SLB was exposed to the laser light for 5 s, and then fluorescence recovery was monitored in real-time and fluorescence micrographs were collected every 1 s for 90 s in total. Lateral diffusion coefficients were obtained by analyzing the fluorescence micrographs, namely the fluorescence intensity as a function of time, based on the Hankel transform method [54].

3.4.5 Minimum Inhibitory Concentration (MIC) Testing

The MIC is defined as the lowest concentration of an antimicrobial agent that inhibits the visual growth of a microorganism [57]. Formally, the MIC value can be explained as the lowest concentration of the agent which exerts bacteriostatic activity. In general, MIC values offer a useful index to assess the susceptibilities of antibacterial

agents and evaluate the scope and potency of new drug candidates. The tests can also be used for studying the emergence of drug-resistant bacterial strains as well. Hence, determining MIC values is typically considered as the first step for evaluating antibacterial efficacy, and is also related to the determination of the minimum bactericidal concentration (MBC). Typically, the agar and broth dilution methods are popular for MIC determination. For the agar dilution method, the defined number of target bacteria is directly loaded on agar plates that already contain the antibacterial agent of interest at different concentrations per plate, and hence a large amount of testing compounds is required. On the other hand, the broth dilution method is appropriate for testing a large number of bacterial species across a broad range of test agents. Specifically, broth microdilution is defined as broth dilution performed by using microtiter plates with a maximum volume of 500 μl per well [58]. Owing to simpler steps and a smaller amount of sample required, broth microdilution is a very popular method. However, since antibacterial agents have varied properties such as solubility and chemical stability, their activity can be varied depending on the testing conditions, and some modification of the assay might be necessary, at least advantageous, depending on the test compound, *e.g.*, the efficacy of certain antimicrobial peptides are inhibited in the presence of Ca^{2+} and Mg^{2+} ions [59, 60]. Mueller–Hinton (MH) agar and broth are standard media recommended by the Clinical and Laboratory Standards Institute (CLSI) [61] in the United States and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [62] for antibacterial susceptibility testing on a wide spectrum of nonfastidious microorganisms such as *Staphylococcus* species. As presented in **Figure 3.18**, microdilution is usually performed with a serial dilution of test compounds in culture medium, followed by the addition of a bacterial inoculum with defined cell number to the solutions containing diluted compound. After incubation for a certain period of time, MIC values can be read by recording the optical turbidity of each well. The concentration of test agent at which there is no change in turbidity signifies the MIC value. Alternatively, it is possible to use a modified version of the MIC assay whereby bacteria and test compound are incubated in a certain medium (*e.g.*, PBS) for a certain incubation time, usually on the scale of hours, and then bacteria are plated onto agar plates for MIC determination based on visual detection of bacterial cell colonies. The

Figure 3.18 Schematic presentation of MIC assay format using microtiter plates and layout of experimental designs.

agar allows the bacteria to grow rapidly, while bacteria grow much more slowly in PBS. In this thesis, the modified approach for antibacterial testing was chosen because it permitted the evaluation of antimicrobial lipids in PBS solution and hence the concentration-dependent molecular self-assembly of the test compounds mirrored the self-assembly behavior observed in the biophysical assays (which were conducted in equivalent PBS solution). Furthermore, selected experiments were conducted across the broth microdilution and modified methods in order to validate the determined MIC values.

Experimental Protocol:

MIC values were determined under different solution conditions against standard *S. aureus* and methicillin-resistant *S. aureus* (MRSA). In Chapter 4, the tests were conducted in PB, PBS and MH broth, while the preliminary tests in Chapter 7 were conducted in PBS alone. As mentioned above, in most experiments, the MIC method used in this thesis was modified based on matching the conditions used in biophysical assays and to improve the solubility of fatty acids and monoglycerides in aqueous condition (along with minimizing nonspecific binding with matrix components). When using PB or PBS buffer, serial twofold dilutions of the test compound solution, containing the fatty acid or monoglyceride or mixture therefore, were prepared in 96-well microtiter plates. 50 μ l of the bacterial cell culture at 1×10^6 CFU/ml were mixed together with an equal volume of diluted compound solution in each well, and the final

cell density was 5×10^5 CFU/ml. The bacterial-compound mixture was incubated for 3 hrs at 37 °C and a 10 µl volume was then streaked onto MH agar plates by using a sterile loop. The streaked agar plates were incubated for 24 hrs at 37 °C, and then the MIC values were determined at the lowest concentration of test compound that inhibits growth of the bacterial colonies. In case of using MH broth, 75 µl of the test compound was mixed with an equal volume of bacterial cell culture and incubated with shaking for 24 hrs at 37 °C. After the incubation period, the MIC values were measured by OD₆₀₀ measurements using a microplate reader.

References

- [1] R.A. Durst, B.R. Staples, *Clinical Chemistry*, 18 (1972) 206-208.
- [2] V.S. Stoll, J.S. Blanchard, *Methods in Enzymology*, 182 (1990) 24-38.
- [3] J. Sambrook, D.W. Russell, *Cold Spring Harbor Laboratory Press*, New York, (2001).
- [4] S.R. Tabaei, J.-H. Choi, G. Haw Zan, V.P. Zhdanov, N.-J. Cho, *Langmuir*, 30 (2014) 10363-10373.
- [5] S.R. Tabaei, J.A. Jackman, M. Kim, S. Yorulmaz, S. Vafaei, N.-J. Cho, *Journal of Visualized Experiments: JoVE*, (2015).
- [6] S.R. Tabaei, J.A. Jackman, S.-O. Kim, B. Liedberg, W. Knoll, A.N. Parikh, N.-J. Cho, *Langmuir*, 30 (2014) 13345-13352.
- [7] V.E. Bower, M. Paabo, R. Bates, *Journal of Research of the National Bureau of Standards*, 65 (1961) 267-270.
- [8] T.J. Treangen, R.A. Maybank, S. Enke, M.B. Friss, L.F. Diviak, D.K. Karaolis, S. Koren, B. Ondov, A.M. Phillippy, N.H. Bergman, *Genome Announcements*, 2 (2014) e01110-01114.
- [9] F. Widdel, *Di dalam Grundpraktikum Mikrobiologie*, 4 (2007).
- [10] H. Vorum, R. Brodersen, U. Kragh-Hansen, A.O. Pedersen, *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 1126 (1992) 135-142.
- [11] C.-H. Chen, Y. Wang, T. Nakatsuji, Y.-T. Liu, C. Zouboulis, R.L. Gallo, L. Zhang, M.-F. Hsieh, C.-M. Huang, *Journal of Microbiology and Biotechnology*, 21 (2011) 391-399.
- [12] S. Thamphiwatana, W. Gao, M. Obonyo, L. Zhang, *Proceedings of the National Academy of Sciences*, 111 (2014) 17600-17605.
- [13] M. Obonyo, L. Zhang, S. Thamphiwatana, D. Pornpattananangkul, V. Fu, L. Zhang, *Molecular Pharmaceutics*, 9 (2012) 2677-2685.
- [14] M.-H. Schmid, H. Korting, *Advanced Drug Delivery Reviews*, 18 (1996) 335-342.
- [15] J.J. Kabara, D.M. Swieczkowski, A.J. Conley, J.P. Truant, *Antimicrobial Agents and Chemotherapy*, 2 (1972) 23-28.
- [16] P. Peterlin, V. Arrigler, K. Kogej, S. Svetina, P. Walde, *Chemistry and Physics of Lipids*, 159 (2009) 67-76.

- [17] M. Mally, P. Peterlin, S.a. Svetina, *The Journal of Physical Chemistry B*, 117 (2013) 12086-12094.
- [18] K. Giger, E.R. Lamberson, J.S. Hovis, *Langmuir*, 25 (2008) 71-74.
- [19] E. Sackmann, *Science-AAAS-Weekly Paper Edition*, 271 (1996) 43-48.
- [20] E. Kalb, S. Frey, L.K. Tamm, *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1103 (1992) 307-316.
- [21] M.D. Mager, N.A. Melosh, *Langmuir*, 23 (2007) 9369-9377.
- [22] E. Evans, W. Rawicz, *Physical Review Letters*, 64 (1990) 2094.
- [23] A.O. Hohner, M.P.C. David, J.O. Rädler, *Biointerphases*, 5 (2010) 1-8.
- [24] E. Fisicaro, C. Compari, E. Duce, M. Biemmi, M. Peroni, A. Braibanti, *Physical Chemistry Chemical Physics*, 10 (2008) 3903-3914.
- [25] A. Domínguez, A. Fernández, N. González, E. Iglesias, L. Montenegro, *Journal of Chemical Education*, 74 (1997) 1227.
- [26] E. Goddard, N. Turro, P. Kuo, K. Ananthapadmanabhan, *Langmuir*, 1 (1985) 352-355.
- [27] J.W. Lichtman, J.-A. Conchello, *Nature Methods*, 2 (2005) 910.
- [28] K. Kalyanasundaram, J. Thomas, *Journal of the American Chemical Society*, 99 (1977) 2039-2044.
- [29] M. Ashaduzzaman, M. Kunitake, *International Letters of Chemistry, Physics and Astronomy*, 1 (2013) 55-62.
- [30] C. Malmberg, A. Maryott, *Journal of Research of the National Bureau of Standards*, 56 (1956) 1-8.
- [31] I. Benito, M. Garcia, C. Monge, J. Saz, M. Marina, *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 125 (1997) 221-224.
- [32] E. Fuguet, C. Ràfols, M. Rosés, E. Bosch, *Analytica Chimica Acta*, 548 (2005) 95-100.
- [33] M. Rodahl, F. Höök, A. Krozer, P. Brzezinski, B. Kasemo, *Review of Scientific Instruments*, 66 (1995) 3924-3930.
- [34] M. Rodahl, F. Höök, C. Fredriksson, C.A. Keller, A. Krozer, P. Brzezinski, M. Voinova, B. Kasemo, *Faraday Discussions*, 107 (1997) 229-246.
- [35] M. Rodahl, B. Kasemo, *Review of Scientific Instruments*, 67 (1996) 3238-3241.

- [36] G. Sauerbrey, *Zeitschrift für Physik A Hadrons and Nuclei*, 155 (1959) 206-222.
- [37] E. Reimhult, C. Larsson, B. Kasemo, F. Höök, *Analytical Chemistry*, 76 (2004) 7211-7220.
- [38] M.C. Dixon, *Journal of Biomolecular Techniques: JBT*, 19 (2008) 151.
- [39] M. Voinova, M. Jonson, B. Kasemo, *Biosensors and Bioelectronics*, 17 (2002) 835-841.
- [40] N.-J. Cho, C.W. Frank, B. Kasemo, F. Höök, *Nature Protocols*, 5 (2010) 1096-1106.
- [41] D. Coling, B. Kachar, *Current Protocols in Neuroscience*, (1997) 2.1. 1-2.1. 11.
- [42] D. Axelrod, *Traffic*, 2 (2001) 764-774.
- [43] J. Pawley, B.R. Masters, *Optical Engineering*, 35 (1996) 2765-2766.
- [44] H. Matsumoto, S. Kitamura, T. Araki, *Frontiers of Medical and Biological Engineering*, 10 (2000) 269-284.
- [45] P. Meyer, J. Dworkin, *Research in Microbiology*, 158 (2007) 187-194.
- [46] S. Yorulmaz Avsar, J.A. Jackman, M.C. Kim, B.K. Yoon, W. Hunziker, N.-J. Cho, *Langmuir*, 33 (2017) 7332-7342.
- [47] M.C. Kim, A. Gunnarsson, S.R. Tabaei, F. Höök, N.-J. Cho, *Physical Chemistry Chemical Physics*, 18 (2016) 3040-3047.
- [48] D. Thid, J.J. Benkoski, S. Svedhem, B. Kasemo, J. Gold, *Langmuir*, 23 (2007) 5878-5881.
- [49] D. Soumpasis, *Biophysical Journal*, 41 (1983) 95-97.
- [50] H. Ishikawa-Ankerhold, R. Ankerhold, G. Drummen, *Essential for Life Science*, (2001)
- [51] E.L. Cussler, *Diffusion: Mass Transfer in Fluid Systems*, Cambridge University Press, (2009).
- [52] K. Jacobson, A. Ishihara, R. Inman, *Annual Review of Physiology*, 49 (1987) 163-175.
- [53] H. Kapitza, G. McGregor, K. Jacobson, *Proceedings of the National Academy of Sciences*, 82 (1985) 4122-4126.
- [54] P. Jönsson, M.P. Jonsson, J.O. Tegenfeldt, F. Höök, *Biophysical Journal*, 95 (2008) 5334-5348.

- [55] J.A. Jackman, S.R. Tabaei, Z. Zhao, S. Yorulmaz, N.-J. Cho, *ACS Applied Materials & Interfaces*, 7 (2014) 959-968.
- [56] M.D. Mager, B. Almquist, N.A. Melosh, *Langmuir*, 24 (2008) 12734-12737.
- [57] J.M. Andrews, *Journal of Antimicrobial Chemotherapy*, 48 (2001) 5-16.
- [58] I. Wiegand, K. Hilpert, R.E. Hancock, *Nature Protocols*, 3 (2008) 163-175.
- [59] D.M. Bowdish, D.J. Davidson, R. Hancock, *Current Protein and Peptide Science*, 6 (2005) 35-51.
- [60] R.E. Hancock, H.-G. Sahl, *Nature Biotechnology*, 24 (2006) 1551-1557.
- [61] P. Wayne, *Performance Standards for Antimicrobial Susceptibility Testing*, 17 (2007).
- [62] M. European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical, D. Infectious, *Clinical Microbiology and Infection*, 9 (2003) ix-xv.

Chapter 4

Spectrum of Membrane Morphological Responses to Antibacterial Fatty Acids and Related Surfactants*

*Medium-chain saturated fatty acids and related compounds (e.g., monoglycerides) represent one class of membrane-active surfactants with antimicrobial properties. Most related studies have been in vitro evaluations of bacterial growth inhibition, and there is limited knowledge about how these compounds destabilize lipid bilayers, which are the purported target within bacterial cell membranes. Herein, the interaction between three representative compounds in this class and a supported lipid bilayer platform was investigated using the QCM-D and fluorescence microscopy techniques in order to examine membrane destabilization. For each tested compound, striking differences in the resulting morphological changes of supported lipid bilayers were discovered. The experimental trends indicate that the compounds have membrane-disruptive behavior against supported lipid bilayers principally above the respective critical micelle concentration values. The growth inhibition properties of the compounds against standard and methicillin-resistant *Staphylococcus aureus* bacterial strains were also tested. Taken together, the findings improve knowledge about how saturated fatty acids and related compounds destabilize lipid bilayers, offering insight into the corresponding molecular mechanisms that lead to membrane morphological responses.*

*This chapter is published as B.K. Yoon, J.A. Jackman, M.C. Kim, and N.J. Cho. Spectrum of Membrane Morphological Responses to Antibacterial Fatty Acids and Related Surfactants. *Langmuir* **31**, 10223–10232 (2015). DOI: 10.1021/acs.langmuir.5b02088.

4.1 Introduction

Antibiotic resistance is one of the most serious public health issues in the world, sparked in part by the overuse of antibiotics in medicine and agriculture [1]. In the face of multidrug-resistant bacteria, many antibiotics are losing effectiveness and there is growing recognition that a post-antibiotic era is approaching [2]. To address this issue, there has been renewed attention on antimicrobial lipids which act as mild surfactants that damage bacterial cell membranes [3]. While antibiotics interfere with specific processes in the bacterial life cycle (*e.g.*, inhibit enzymes involved in cell wall synthesis) that leave them prone to the evolution of antibiotic-resistant bacterial strains [4], certain lipids, and more broadly surfactants in general, act promiscuously in order to target the bacterial cell membrane and can exhibit bacteriostatic or bactericidal effects [5]. Partial solubilization of the cell membrane interferes with metabolic regulation, leading to inhibition of bacterial growth. On the other hand, membrane lysis irreversibly damages a bacterium and causes cell death, often quickly on the time scale of minutes. Owing to the multiple ways by which antimicrobial lipids target bacterial cell membranes, there is a high barrier to the emergence of drug-resistant bacterial strains [6].

At the same time, the promiscuity of antimicrobial lipids can also have deleterious effects on host cell membranes, and surfactants either in free form [7] or emulsions [8] have been primarily used as topical microbicides or in industrial applications (*e.g.*, food preservatives). Liposomal formulations have also been developed [9-14] in order to encapsulate fatty acids, offering a delivery vehicle that minimizes host cell toxicity and other deleterious side effects while showing therapeutic promise to reduce bacterial loads in *in vitro* and *in vivo* studies, including for both topical and systemic administration routes. Small liposomes loaded with fatty acids in the bilayer can fuse with the bacterial cell membrane, leading to release of high local concentrations of fatty acids into the bacterial cell membrane which results in gross morphological changes and enhanced membrane permeability that causes bacterial cell death [13]. In all the embodiments – free form, emulsion, or liposome – the key contributing factor to antibacterial activity is the antimicrobial lipid itself and there is great interest to understand how lipids and surfactants interfere with bacterial cell membranes.

To date, numerous antimicrobial lipids and structurally related surfactants have been discovered or synthesized with variable efficacies and spectra. In order to understand how the chemical structure of a lipid influences its antimicrobial properties, extensive *in vitro* studies have been conducted and open the door to mechanistic investigations. Kabara and colleagues led pioneering studies on the antibacterial properties of medium-chain saturated fatty acids [15-17]. Through systematic investigation of saturated fatty acids with chain lengths between 6 and 18 carbons, it was determined that lauric acid has the most potent inhibitory activity against Gram-positive bacteria [18]. The 1-monoglyceride derivative of lauric acid is called glycerol monolaurate and was discovered to have a lower minimum inhibitory concentration than lauric acid, albeit against a narrower range of bacteria [19]. Both lauric acid and glycerol monolaurate (GML) are generally recognized as safe by the United States Food and Drug Administration and are used as food preservatives and cosmetic emulsifiers [20]. Another related compound is sodium dodecyl sulfate (SDS) which is widely used as a detergent with known antimicrobial properties [21]. As presented in **Figure 4.1**, all three compounds share a 12-carbon long primary alkyl chain and have different headgroups that influence the corresponding physicochemical properties.

While the structure-activity relationships of fatty acids and related compounds have been investigated in terms of bacterial growth inhibition, it remains to be understood how treatment with these compounds affects the properties of lipid membranes. Indeed, surfactants in general encompass a spectrum of mechanistic behaviors (*e.g.*, permeabilization, lysis, etc.), with the molecular-level details depending on the physicochemical properties and concentration of the surfactant [22]. Thus far, there have been extensive mechanistic studies of cationic amphiphiles, especially membrane-active peptides, but specific modes of action still remain to be defined, in part reflecting the complexity of studying biological systems [23]. Scanning electron microscopy has been employed to visualize fixed specimens of bacteria *after* treatment with high concentrations of certain fatty acids and derivatives [24]. However, there has been no direct investigation of the membrane destabilization process for saturated fatty acids and related compounds.

Figure 4.1 Chemical structure of tested antimicrobial lipids. Lauric acid, glycerol monolaurate, and SDS each have a 12-carbon long primary alkyl chain. The critical micelle concentrations of the surfactants in aqueous solution are: lauric acid (1.5 mM, Ref. [25]); glycerol monolaurate (42 μ M, Ref. [26]); and SDS (260 μ M, Ref. [26]). The quoted CMC values were obtained from literature reports which investigated the surfactants in high ionic strength aqueous conditions comparable to the conditions used in this study.

As a representative model of cell membranes, the supported lipid bilayer platform has proven useful in order to evaluate the interfacial activity of other classes of membrane-active compounds, including phospholipase enzymes [27] and the aforementioned cationic antimicrobial peptides [28]. Importantly, the platform is compatible with a wide range of surface-sensitive measurement techniques such as label-free acoustic and optical sensors as well as fluorescence microscopy and atomic force microscopy techniques which enable detailed examination of lipid bilayer properties *during* treatment with membrane-active compounds [29]. Such capabilities are well-suited to investigate the dynamic processes by which medium-chain saturated fatty acids and related compounds destabilize lipid bilayers.

The goal of the present study was to compare how lauric acid, GML, and SDS affect the physical properties of supported lipid bilayers. Quartz crystal microbalance-dissipation (QCM-D) and fluorescence microscopy experiments were employed in order to monitor changes in the mass, viscoelastic, and morphological properties of the

supported lipid bilayer upon treatment with compounds at various concentrations. This approach provided a quantitative measurement framework in order to compare the interfacial activity of the different compounds. The bacterial growth inhibition properties of each compound was also tested against standard and methicillin-resistant *Staphylococcus aureus* – a Gram-positive bacterium which is a leading cause of antibiotic-resistant infections in humans [30] – by determining minimum inhibitory concentration (MIC) values. Collectively, the findings present evidence that the tested compounds differentially act against supported lipid bilayers, offering insight into how these compounds interfere with lipid membranes and induce membrane morphological changes.

4.2 Materials and Methods

4.2.1 Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Lauric acid, sodium dodecyl sulfate, Mueller-Hinton (MH) agar, and Mueller-Hinton broth were obtained from Sigma Aldrich (St Louis, MO). Glycerol monolaurate was purchased from Abcam (Cambridge, UK). Luria-Bertani (LB) broth was purchased from BD (Sparks, MD). Phosphate-buffered saline (PBS) was purchased from Gibco (Carlsbad, CA). All solutions were prepared with MilliQ-treated water ($>18\text{ M}\Omega\cdot\text{cm}$) (Millipore, Billerica, MA).

4.2.2 Preparation of Antimicrobial Lipid Solutions

The desired mass of antimicrobial lipid powder was weighed using an analytical balance. Stock solutions of lauric acid and GML were prepared by first dissolving the weighed compounds in ethanol to a final concentration of 200 mM. In order to prepare lauric acid and GML test samples, an aliquot from the stock solution was diluted 1:100 with phosphate buffer (PB), phosphate-buffered saline (PBS), or Mueller-Hinton (MH) broth. The concentration of the diluted compound was 2 mM. SDS test samples were

prepared by dissolving the weighed compound in the appropriate media (without ethanol step) to a concentration of 2 mM. To increase the solubility of all test samples, the solutions were heated in a 70°C water bath for 30 min. Subsequent dilutions were made in accordance with the experimental protocols. The maximum concentration of ethanol at the highest test concentration was 1% in aqueous solution and lauric acid ($pK_a \sim 5$) and SDS ($pK_a \sim 2$) are assumed to be deprotonated under all test conditions. All solutions were prepared on the same day as experiments. The dry compounds were stored in a dark cabinet.

4.2.3 Quartz Crystal Microbalance-Dissipation (QCM-D) Experiments

QCM-D experiments with a Q-Sense E4 instrument (Q-Sense AB, Gothenburg, Sweden) were conducted in order to characterize the interaction between the test compounds and a supported lipid bilayer. The QCM-D technique monitors changes in the resonance frequency (Δf) and energy dissipation (ΔD) of an oscillating, piezoelectric quartz crystal sensor chip as functions of time, which reflect the acoustic mass and viscoelastic properties, respectively, of an adsorbate on the surface [31]. The sensor chip had a fundamental frequency of 5 MHz and a sputter-coated, 50-nm thick layer of silicon dioxide (model no. QSX 303, Q-Sense AB). Before experiment, the sensor chips were sequentially rinsed with water and ethanol, dried with nitrogen gas, and subjected to oxygen plasma treatment for 1 min with an Expanded Plasma Cleaner (model no. PDC-002, Harrick Plasma, Ithaca, NY). In the experiments, a supported lipid bilayer comprised of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipid was initially formed using the solvent-assisted lipid bilayer (SALB) technique [32]. Briefly, a baseline signal was recorded in aqueous buffer solution (10 mM Tris, 150 mM NaCl, pH 7.5) and then substituted with isopropanol solution. Next, 0.5 mg/mL DOPC lipid in isopropanol solution was added and allowed to equilibrate for 10 min followed by exchange with aqueous buffer solution in order to form the supported lipid bilayer. Finally, 50 μ M bovine serum albumin (BSA) was added as a blocking agent in order to prevent nonspecific adsorption of antibacterial agents in subsequent steps. After the bilayer formation process was completed, the solution was exchanged to PBS solution and then

the test compound in an identical PBS solution was added under continuous flow conditions for a minimum of 30 min. A washing step with PBS solution completed the procedure. In all steps, a peristaltic pump (Reglo Digital, Ismatec, Glattbrugg, Switzerland) was used to inject liquid samples into the measurement chamber at a flow rate of 50 $\mu\text{L}/\text{min}$. During the experiments, the temperature in the measurement cell was maintained at 25.0 ± 0.5 °C. The experimental data was collected at the 3rd ($n = 3$), 5th ($n = 5$), and 7th ($n = 7$) odd overtones using the QSoft (Q-Sense AB) software package, and the data was normalized according to the overtone number. Data processing was performed in the QTools (Q-Sense AB) and OriginPro 8.5 (OriginLab, Northampton, MA) software packages. All presented data was collected at the 5th overtone.

4.2.4 Fluorescence Microscopy Experiments

Epifluorescence microscopy was performed in order to directly observe morphological changes in supported lipid bilayers due to treatment with the test compounds. An Eclipse TI-U inverted optical microscope (Nikon, Japan) with a 60x magnification (NA=1.49) oil immersion objective lens (Nikon) was used and images were recorded by an iXon 512 x 512 pixel EMCCD camera (Andor Technology, Northern Ireland). The pixel size was 0.267×0.267 μm . A fiber-coupled mercury lamp (Intensilight C-HGFIE, Nikon) was used to illuminate the fluorophores with a TRITC filter. Initially, supported lipid bilayers were formed by the vesicle fusion method (0.2 mg/mL extruded DOPC lipid vesicles with 68 nm diameter; see Ref. [33]) inside a microfluidic flow-through chamber (Sticky slide VI 0.4, Ibidi, Germany). After formation, the lipid bilayer was rinsed with buffer solution and then the test compound was introduced into the measurement chamber at a flow rate of 40 $\mu\text{L}/\text{min}$. During this stage, time-lapsed images were recorded every 5 sec for a total duration of 30 min. The initial time, $t = 0$ sec, was defined by when the test compound solution reached the channel inlets. For each measurement recording, the fluorescence intensity of each image was normalized using a custom-written script for the Python(x,y) 2.7.5 software program.

4.2.5 Bacterial Cell Culture

S. aureus (ATCC 25923) and methicillin-resistant *S. aureus* (ATCC 33591) (American Type Culture Collection, Manassas, VA) were cultured in LB broth overnight at 37°C. The suspension of the overnight culture was inoculated in fresh LB broth and cultured under aerobic conditions until reaching an OD₆₀₀ value of approximately 0.5 (exponential growth phase). The bacterial cells were harvested by centrifugation at 1,500 x g for 10 min, washed thrice with PB or PBS, and re-suspended in the appropriate solution for experiment. The bacterial cell suspensions were diluted to an OD₆₀₀ value of 0.1, which corresponds to 1×10^7 CFU/mL for *S. aureus* [34]. Before experiment, the bacterial cells were then diluted 1:10 with PB, PBS, or MHB.

4.2.6 Minimum Inhibitory Concentration (MIC) Testing

The MIC values of the test compounds were determined in PB, PBS, and MH broth solutions [35]. For the PB and PBS cases, the MIC determination was made by a MH agar plate method. Two-fold serial dilutions of test compound solutions in PB or PBS were first made in a 96-well microtiter plate. The test compound concentration ranged from 8 µM to 1 mM with 50 µL volume. Then, 50 µL of *S. aureus* (1×10^6 CFU/mL) suspended in PB or PBS was added to each well for a final cell density of 5×10^5 CFU/mL. The samples were incubated for 3 hr at 37°C, and then streaked onto MH agar plates. The plates were incubated overnight at 37°C and the MIC value was recorded based on the lowest test compound concentration that inhibited colony growth. For the MH broth case, the MIC determination was made by the broth microdilution method. Two-fold serial dilutions of test compound solutions in MH broth were first made in a 96-well microtiter plate. The test compound concentration ranged from 4 µM to 2 mM with 75 µL volume. Then, 75 µL of *S. aureus* (1×10^6 CFU/mL) suspended in MH broth was added to each well for a final cell density of 5×10^5 CFU/mL. The samples were incubated with shaking for 24 hr at 37°C. After incubation, the MIC value was recorded by OD₆₀₀ measurement using a microplate reader.

4.3 Results

4.3.1 Effect on Mass and Viscoelastic Properties of Supported Lipid Bilayers

To investigate the effect of the test compounds on lipid bilayers, supported lipid bilayers on silicon dioxide surfaces were initially formed using the solvent-assisted lipid bilayer (SALB) formation method [32]. The single-component lipid bilayers were made of zwitterionic DOPC lipid. The QCM-D technique was employed in order to monitor the bilayer fabrication process and the experimental protocol consistently yielded supported lipid bilayers with final Δf and ΔD shifts of -26 ± 2 Hz and $0.3 \pm 0.2 \times 10^{-6}$, respectively. These values are in good agreement with expected values for a supported lipid bilayer [36] and further confirmed by a bovine serum albumin (BSA) blocking step. BSA protein exhibits significant adsorption onto hydrophilic silicon dioxide, but not onto zwitterionic lipid bilayers [37]. A large reduction in BSA adsorption to the bilayer-coated substrates was observed in comparison to bare substrates, supporting that the lipid bilayers form homogenous and complete ($> 95\%$) thin films. After fabrication, the supported lipid bilayers were exposed to a flow-through sample of test compound with a fixed bulk concentration, as monitored by the QCM-D technique. The measurement results obtained for each compound are described below. In Figures 2-4, note that the initial baseline values for the Δf and ΔD shifts correspond to supported lipid bilayer formation.

4.3.1.1 Sodium Dodecyl Sulfate

Figure 4.2 presents the effects of SDS on the Δf and ΔD shifts as a function of SDS concentration. At 1 mM and higher SDS concentrations, there was a rapid decrease in Δf to around -45 Hz that was mirrored by an increase in ΔD to around 9×10^{-6} (**Figure 4.2A,B**). The transient spikes were quickly diminished, leading to final Δf and ΔD values around -4 Hz and 1.5×10^{-6} , respectively. A washing step entirely removed lipid and SDS from the substrate, as indicated by final Δf and ΔD values around 0 Hz and 0×10^{-6} , respectively.

At lower SDS concentrations between 125 μM and 500 μM , similar initial decreases in Δf were observed around -47 Hz and the time required to reach the inflection point was inversely proportional to the bulk SDS concentration (**Figure 4.2C-E**). The stabilized Δf values nearly stabilized around -30 Hz to -39 Hz over a longer time. On the

Figure 4.2 QCM-D Investigation of SDS treatment on supported lipid bilayers. Δf (blue line with squares) and ΔD (red line with triangles) shifts as functions of time are presented for (A) 2 mM, (B) 1 mM, (C) 500 μM , (D) 250 μM , (E) 125 μM , and (F) 63 μM SDS. The critical micelle concentration of SDS is 260 μM [26]. The initial measurement values correspond to a supported lipid bilayer on the silicon dioxide surface. SDS was added at $t = 5$ min (arrow 1) and a washing step was performed (arrow 2) after the measurement signals stabilized.

other hand, in this concentration range, the ΔD shift did not correlate with the Δf shift. Rather, there was a monotonic increase in ΔD that weakly increased with SDS concentration, from 5×10^{-6} at 125 μM to 8×10^{-6} at 500 μM . Upon washing, the Δf values increased to around -22 to -25 Hz, and the ΔD values decreased to between 1×10^{-6} and 4×10^{-6} .

At 63 μM SDS, there was a negligible change in Δf and a minor increase in ΔD to around 3×10^{-6} (**Figure 4.2F**). A washing step led to a moderate increase in Δf to approximately -14 Hz and negligible change in the ΔD value. Taken together, 1 mM and higher SDS concentrations induced complete solubilization of the lipid bilayer through a transient stage of membrane perturbation, while lower SDS concentrations induced changes in the lipid bilayer without complete solubilization.

4.3.1.2 Lauric Acid

Figure 4.3 presents the effects of lauric acid on the Δf and ΔD shifts as a function of lauric acid concentration. At 2 mM lauric acid concentration, there was a simultaneous decrease in Δf and increase in ΔD to -42 and 7×10^{-6} , respectively (**Figure 4.3A**). The Δf shift returned upwards to around -31 Hz while the ΔD shift decreased and stabilized around 3×10^{-6} . A washing step led to the Δf and ΔD shifts reaching -27 Hz and 2×10^{-6} , respectively. At 1 mM lauric acid concentration, there was a minor decrease in Δf to -31 Hz and an increase in ΔD to 3×10^{-6} (**Figure 4.3B**). A washing step led to an increase in Δf to -27 Hz and a decrease in ΔD to 1.2×10^{-6} . Similar, although markedly attenuated, results were observed at 500 μM lauric acid and there were negligible changes at 250 μM lauric acid (**Figure 4.3C,D**). Hence, lauric acid affected the measured properties of the lipid bilayer only at high concentrations and lower concentrations had minimal effects on the lipid bilayer.

Figure 4.3 QCM-D Investigation of lauric acid treatment on supported lipid bilayers. Δf (blue line with squares) and ΔD (red line with triangles) shifts as functions of time are presented for (A) 2 mM, (B) 1 mM, (C) 500 μ M, and (D) 250 μ M lauric acid. The critical micelle concentration of lauric acid is 1.5 mM [25]. The initial measurement values correspond to a supported lipid bilayer on the silicon dioxide surface. Lauric acid was added at $t = 5$ min (arrow 1) and a washing step was performed (arrow 2) after the measurement signals stabilized.

4.3.1.3 Glycerol Monolaurate

Figure 4.4 presents the effects of GML on the Δf and ΔD shifts as a function of GML concentration. At 2 mM GML, there was a rapid drop in Δf to -59 Hz and corresponding increase in ΔD to 21×10^{-6} . The Δf and ΔD values stabilized around -33 Hz and 8×10^{-6} , respectively (**Figure 4.4A**). A washing step led to an increase in the Δf shift to -5 Hz and a decrease in the ΔD shift to around 0×10^{-6} . By contrast, at 1 mM GML, a more gradual but appreciable decrease in Δf reached -118 Hz before incrementally rising to around -90 Hz (**Figure 4.4B**). The corresponding ΔD shift

increased monotonically to approximately 48×10^{-6} . A washing step led to a pronounced decrease in Δf to around -145 Hz before quickly increasing to -12 Hz. In this stage, the ΔD shift more closely paralleled the Δf shift with an increase to 56×10^{-6} followed by a decrease to 2×10^{-6} . Similar trends were observed down to 250 μM GML with even larger Δf and ΔD shifts reaching up to -180 Hz and 64×10^{-6} , respectively (**Figure 4.4C,D**). At high bulk lipid concentrations, some degree of membrane degradation can occur due to membrane lysis.

Figure 4.4 QCM-D Investigation of GML treatment on supported lipid bilayers. Δf (blue line with squares) and ΔD (red line with triangles) shifts as functions of time are presented for (A) 2 mM, (B) 1 mM, (C) 500 μM , (D) 250 μM , (E) 125 μM , (F) 63 μM , (G) 31 μM , (H) 16 μM , and (I) 8 μM GML. The critical micelle concentration of GML is 42 μM [26]. The initial measurement values correspond to a supported lipid bilayer on the silicon dioxide surface. GML was added at $t = 5$ min (arrow 1) and a washing step was performed (arrow 2) after the measurement signals stabilized.

At lower concentrations, the effect of GML on lipid bilayers was less pronounced but still significant. At 125 μM GML, there were simultaneous Δf and ΔD shifts that stabilized around -108 Hz and 34×10^{-6} , respectively (**Figure 4.4E**). A washing step reduced the Δf and ΔD shifts to final values of -10 Hz and 1×10^{-6} , respectively. Between 63 and 16 μM GML, appreciably smaller effects on the lipid bilayer were detected (**Figure 4.4F-H**). The Δf and ΔD shifts reached up to -32 Hz and 4×10^{-6} , respectively, largely independent of GML concentration in this regime. Washing steps indicated that the final Δf and ΔD shifts were minor. At 8 μM GML, there was no discernible effect on the lipid bilayer (**Figure 4.4I**). Taken together, the results indicate that GML has pronounced activity at 125 μM and higher, moderate activity between 63 and 16 μM , and minimal activity at 8 μM and below.

4.3.2 Observation of Morphological Changes in Supported Lipid Bilayers

In order to visualize morphological changes in the supported lipid bilayer, fluorescence microscopy experiments were also performed. The lipid composition was 99.5 mol% DOPC lipid and 0.5 mol% 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) lipid. The experiments were conducted in a flow-through microfluidic chamber in order to facilitate the addition of test compounds, as followed by fluorescence microscopy with time-lapsed recording. Note that $t = 0$ sec corresponds to the time when the test compound reached the measurement chamber. Based on the QCM-D measurements, specific concentrations of each compound were selected for further investigation based on the conditions which yielded significant changes in the measured properties of the lipid bilayers. The selected concentrations were 2 mM for SDS and lauric acid, and 500 μM for GML. The experimental results obtained for each compound are described below.

4.3.2.1 Sodium Dodecyl Sulfate

As shown in **Figure 4.5**, tubule formation followed by bilayer solubilization was induced by SDS. Bright spots became visible on the lipid bilayer within 15 sec, and depict nucleation sites for tubular protrusions. The time scale of protrusion growth varied

Figure 4.5 Microscopic observation of SDS-induced solubilization of supported lipid bilayers. (A-I) Image snapshots at various time points during SDS introduction depict nucleation sites that promote tubule formation followed by complete solubilization of the lipid bilayer. $t = 0$ sec corresponds to the introduction of 2 mM SDS solution into the measurement chamber. The scale bars are 20 μm .

significantly, with some fibrils quickly growing to over 20 μm length within 75 sec while other fibrils did not grow appreciably. Due to the continuous flow conditions, the tubules were oriented in one direction and some continued to grow. In due course, there was a pronounced decrease in the fluorescence intensity on the substrate indicative of lipid removal, and complete solubilization of the lipid bilayer – indicated by the lack of fluorescence intensity on the substrate – was observed within 10 min.

4.3.2.2 Lauric Acid

Figure 4.6 demonstrates that lauric acid also induces the tubule formation on the lipid bilayer. Bright spots were visible within 50 sec and represent nucleation sites for the tubular protrusions, similar to the SDS case. Again, due to the flow conditions, the tubules were oriented in one direction. A dense coverage of tubules was reached within 5 min. With increasing time, the number of tubules decreased, likely due to the detachment of some tubules from the lipid bilayer. However, complete solubilization of the lipid bilayer was not observed. Instead, most tubules remained stably attached for greater than 30 min. After the flow was stopped, the parallel orientation of the tubules was lost, and replaced by an out-of-plane orientation. Upon a buffer wash, nearly all tubular protrusions were removed from the lipid bilayer and numerous defects (or phase-separated domains) in the lipid bilayer were apparent, as denoted by fluorophore-poor regions which persisted over time.

Figure 4.6 Microscopic observation of lauric acid-induced tubule formation on supported lipid bilayers. (A-F) Image snapshots at various time points depict nucleation sites from which stable tubules grow. $t = 0$ sec corresponds to the introduction of 2 mM lauric acid solution into the measurement chamber. (G) Image snapshot after the flow was stopped. (H-I) Image snapshots show the effects of buffer rinsing, including tubule lysis and appearance of fluorophore-poor domains. The scale bars are 20 μm .

4.3.2.3 Glycerol Monolaurate

In contrast to the morphological changes induced by SDS and lauric acid, GML incorporation led to the formation of spherical protrusions in lipid bilayers, as presented in **Figure 4.7**. Within 10 secs, nucleation of tubular protrusions was initially observed. However, the tubules were not oriented in the flow direction. Rather, the individual tubules became self-entangled and, in some cases, entangled with adjacent tubules as well. Within 2 min, the entangled tubules disappeared and numerous expanding spherical protrusions were visible. More spherical protrusions formed and coalesced. After the flow was stopped, the spherical protrusions remained stable for at least 10 min. Upon a buffer wash, many of the spherical protrusions were removed from the lipid bilayer within 8 min, although some bright spots remained. With continued washing, fluorophore-poor regions became visible in the lipid bilayer. After the buffer wash was stopped, the fluorophore-poor regions quickly disappeared within 2 min, resulting in uniform fluorescence intensity in the surrounding vicinity. This observation lends credence to the possibility that, in this case, the fluorophore-poor regions correspond to GML-rich domains which mix with other components in the lipid bilayer – in effect, demonstrating self-healing properties. It should be noted, however, that numerous bright spots remained stable and did not change over time.

Figure 4.7 Microscopic observation of GML-induced spherical protrusions on supported lipid bilayers. (A-F) Image snapshots at various time points depict nucleation sites from which entangled tubules grow and form spherical caps. $t = 0$ sec corresponds to the introduction of 500 μM GML solution into the measurement chamber. (G) Image snapshot after the flow was stopped. (H-I) Image snapshots show the effects of buffer rinsing, including cap lysis as well as the appearance and disappearance of fluorophore-poor regions. The scale bars are 20 μm .

4.3.3 Microbicidal Activity against *S. aureus* Bacteria

To further investigate how the surfactant concentration influences microbicidal activity, MIC determinations were conducted in different solution conditions against one *S. aureus* bacterial strain (ATCC 25923) (**Table 4.1**). In low-salt PB solution, MIC values of 500 μM and 1 mM were recorded for SDS and lauric acid, respectively, while GML had a variable MIC value in the range of 15-63 μM depending on the individual experiment. In high-salt PBS solution, both SDS and lauric acid maintained similar MIC values. The MIC value of GML was 31 μM . Finally, MIC values were also recorded in Mueller-Hinton (MH) broth, which is a growth medium for microorganisms. In this case, the MIC value for GML was 125 μM while lauric acid and SDS had values of 500 μM and 1 mM, respectively. The MIC values obtained in MH broth solution agree well with reported literature values and trends (see, *e.g.*, Ref. [18]). Similar results in MH broth were also obtained with a methicillin-resistant *S. aureus* strain (ATCC 33591). Consistent with the supported lipid bilayer experiments, the MIC values of GML were lower than those of lauric acid and SDS, supporting that GML is a more potent membrane interacting compound.

Table 4.1 MIC values for antimicrobial lipids against *S. aureus* bacteria. The MIC values are reported from a minimum of three independent experiments using a two-fold dilution protocol.

Condition	Lauric Acid	SDS	GML
PB Solution ¹	1 mM	500 μM	15-63 μM
PBS Solution ¹	1 mM	500 μM	31 μM
MH Broth ¹	500 μM	1 mM	125 μM
MH Broth ²	250 μM	1 mM	125 μM

¹Standard *S. aureus* bacterial strain (ATCC 25923). ²Methicillin-resistant *S. aureus* bacterial strain (ATCC 33591). Unless otherwise noted, identical results were obtained in all experiments. The only exception was GML experiments in PB solution for which there were variable results between 15 and 63 μM ($36 \pm 24 \mu\text{M}$).

4.4 Discussion

4.4.1 Supported Lipid Bilayer as an Experimental Platform

An experimental objective of this work was to utilize a model system in order to directly compare the effects of three antimicrobial lipids on membrane properties. Previous studies have investigated the antibacterial properties of all three test compounds by determining MIC values obtained in *in vitro* experiments, although there are outstanding questions about how factors such as the bacterial strain and solubilization conditions influence the measurement readout. For example, the recorded MIC value of lauric acid against *S. aureus* bacterial strains varies by one order of magnitude in the literature [38]. This variability in turn limits quantitative comparison of literature MIC values for different antimicrobial lipids.

To address this issue, supported lipid bilayers were employed as a standardized experimental platform that can be investigated with a wide range of surface-sensitive measurement techniques. QCM-D experiments were conducted in order to determine the effect of test compounds on the mass and viscoelastic properties of the supported lipid bilayer, while fluorescence microscopy experiments provided information about morphological changes arising from treatment with the test compounds. For each compound, the two techniques showed excellent agreement in the measurement readouts and distinguished specific molecular-level behaviors, which varied considerably. SDS addition led to an initially large dissipation shift accompanied by a more moderate frequency shift that is consistent with the formation of out-of-plane tubule formations, as previously reported [39], and both techniques also provided conclusive evidence for eventually complete solubilization of the lipid bilayer. Similar trends in tubule formation were observed with lauric acid addition as well, albeit in different concentration ranges and *without* subsequent membrane solubilization. The behavior of lauric acid is similar to that observed with docosahexaenoic acid – a 22-carbon long polyunsaturated fatty acid – when added to supported lipid bilayers [40]. On the other hand, GML addition led to striking differences in the measurement responses, with large frequency shifts and even larger dissipation shifts that are consistent with the formation of spherical caps protruding

from the lipid bilayer. Taken together, the supported lipid bilayer investigations demonstrate that each of the three test compounds has a specific mechanism of action for membrane disruption.

4.4.2 Physicochemical Basis for Morphological Changes

In order to explain why the different fatty acids and derivatives have variable effects on the lipid bilayer morphology, one must take into account the physicochemical properties of the compounds. While all three compounds have a 12-carbon long primary alkyl chain, lauric acid and SDS are anionic surfactants whereas GML is a nonionic surfactant. After amphiphilic compounds insert into the outer leaflet of the bilayer, the compounds can equilibrate by translocating into the inner leaflet [3]. For nonionic surfactants, the time scale is typically rapid (milliseconds to seconds), while the time scale is appreciably longer (hours to days) for charged surfactants [41]. Asymmetric insertion gives rise to a monolayer curvature strain [3], leading to mechanical failure that can cause membrane morphological changes.

In another study, a general mechanical model was recently proposed by Staykova *et al.* in order to describe how confined lipid bilayers passively regulate stress by nucleating and evolving protrusions of varying geometries [42]. With increasing membrane strain, the protrusions formed shift from tubes to caps. The general trends predicted by this model are consistent with the present experimental observations that GML insertion induces less membrane strain than the tested anionic surfactants. Another observation of coalescence of small buds into larger ones is also consistent with tension relief [42]. On the other hand, lauric acid and SDS insertion induces particularly high membrane strain due to slow translocation of the surfactant. SDS has greater positive spontaneous curvature than lauric acid due to a larger headgroup [43], which is consistent with membrane solubilization only in the case of SDS treatment. Overall, the experimentally observed trends in membrane morphological changes are strongly correlated with the physicochemical properties of the surfactants, and support that saturated fatty acids and related compounds can elicit a range of membrane perturbations depending on the molecular properties.

4.4.3 Role of Surfactant Concentration on Membrane Disruption

Another observation reported in this work is that all three tested compounds principally disturb supported lipid bilayers at or above the respective critical micelle concentration (CMC). This observation helps to explain why GML causes gross morphological changes in supported lipid bilayers at lower concentrations than lauric acid and SDS. Importantly, the observation also leads to the key finding that the concentration of test compound influenced the extent of membrane disruption in the supported lipid bilayer platform but did not affect the specific mechanism of action. While SDS induced complete membrane lysis resulting in structural disintegration, lauric acid and GML did not fully lyse the supported lipid bilayer. Hence, the range of membrane-lytic behaviors varied among the tested compounds although the nonspecific mechanism of action against lipid membranes in general should be taken into account when considering application possibilities.

It should be emphasized that the QCM-D experiments were performed under continuous flow conditions so the effects of compound concentration primarily relate to the aggregation state of the compound rather than the total amount of compound in the system. The addition of compounds at bulk concentrations below the corresponding CMC value appears to be insufficient to induce significant changes in membrane morphology even at longer time scales. This observation is consistent with a previous report by Thid *et al.* [40], which supports that the micellar form is important for membrane disruption by the tested compounds. It was speculated that the micellar form provides surfactant aggregates which are needed to facilitate membrane remodeling or to serve as a reservoir source of monomers. In other studies, it has been shown that fatty acids encapsulated in liposomes destabilize bacterial cell membranes. Taken together, existing studies support that surfactant aggregation is important for membrane destabilization and suitable formulations have been devised either spontaneously (micelles forming above the CMC, as in the present study) or through encapsulation (embedded in liposome bilayers).

In this regard, the membrane charge selectivity of the tested compounds also merits attention in the context of understanding the biological relevance of the experimentally observed membrane morphological responses. In the present study, the

supported lipid bilayer was composed of single-component zwitterionic DOPC lipids in order to present a simplified and well-controlled model system. The results prove insightful in order to distinguish various types of morphological changes in response to the antimicrobial lipids. Looking forward, it is important to further understand how the tested compounds and other membrane-active antimicrobials act against bacterial and human cell membranes. Both bacterial and human cell membranes exhibit complex structures and are composed of multiple types of zwitterionic and anionic lipids along with sterols (see Ref. [23] and references therein). In general, bacterial cell membranes are regarded as possessing greater anionic character while the outer leaflet of the human cell plasma membranes is primarily zwitterionic. In some cases, cationic antimicrobials exhibit selective targeting of bacterial cell membranes on the basis of charge selectivity. Taking into account that the tested compounds are either zwitterionic or anionic, membrane charge selectivity is not expected. Nevertheless, the interaction of fatty acids and monoglycerides using supported lipid bilayers with more complex membrane compositions, including the presence of sterols which influence membrane rigidity as presented in Chapter 6, could provide deeper insight into the extent of membrane morphological changes in biologically relevant lipid membranes. Furthermore, it is important to explore delivery vehicles such as liposomes (*cf.* Introduction) and solid lipid nanoparticles [44] in order to encapsulate fatty acids and reduce potential human cell toxicities for application purposes.

4.5 Conclusion

In this chapter, the interactions between a series of membrane-active surfactants – lauric acid, SDS, and GML – and supported lipid bilayers were investigated. The combination of QCM-D and fluorescence microscopy experiments led to the identification that each compound induces membrane morphological changes through a distinctive process which involves the formation of spherical or tubular protrusions out of the lipid bilayer. The measurement results support that the tested compounds induce membrane morphological responses primarily above the respective CMC values. In alignment with the activity testing against the SLB platforms, the measured MIC values

of each compound against standard and methicillin-resistant *S. aureus* bacterial strains further suggest that the compounds inhibit bacterial growth *via* membrane lysis. Taken together, the experimental findings demonstrate that the tested membrane-active surfactants can induce a spectrum of membrane morphological changes in supported lipid bilayers. Future work aimed at extending the scope of tested compounds and the biological relevance of the supported lipid bilayer platform could further improve understanding of how membrane-active surfactants act against bacterial cell membranes and other lipid membranes.

References

- [1] R. Laxminarayan, A. Duse, C. Wattal, A.K. Zaidi, H.F. Wertheim, N. Sumpradit, E. Vlieghe, G.L. Hara, I.M. Gould, H. Goossens, *The Lancet Infectious Diseases*, 13 (2013) 1057-1098.
- [2] A.J. Alanis, *Archives of Medical Research*, 36 (2005) 697-705.
- [3] H. Heerklotz, *Quarterly Reviews of Biophysics*, 41 (2008) 205-264.
- [4] J. Davies, D. Davies, *Microbiology and Molecular Biology Reviews*, 74 (2010) 417-433.
- [5] A.P. Desbois, V.J. Smith, *Applied Microbiology and Biotechnology*, 85 (2010) 1629-1642.
- [6] K.A. Hammer, C.F. Carson, *Antibacterial and Antifungal Activities of Essential Oils*, John Wiley & Sons, Ltd, (2011) 255-306.
- [7] K.L. Strandberg, M.L. Peterson, Y.-C. Lin, M.C. Pack, D.J. Chase, P.M. Schlievert, *Antimicrobial Agents and Chemotherapy*, 54 (2010) 597-601.
- [8] H. Thormar, H. Hilmarsson, G. Bergsson, *Applied and Environmental Microbiology*, 72 (2006) 522-526.
- [9] D. Yang, D. Pornpattananangkul, T. Nakatsuji, M. Chan, D. Carson, C.-M. Huang, L. Zhang, *Biomaterials*, 30 (2009) 6035-6040.
- [10] C.-M. Huang, C.-H. Chen, D. Pornpattananangkul, L. Zhang, M. Chan, M.-F. Hsieh, L. Zhang, *Biomaterials*, 32 (2011) 214-221.
- [11] M. Obonyo, L. Zhang, S. Thamphiwatana, D. Pornpattananangkul, V. Fu, L. Zhang, *Molecular Pharmaceutics*, 9 (2012) 2677-2685.
- [12] D. Pornpattananangkul, V. Fu, S. Thamphiwatana, L. Zhang, M. Chen, J. Vecchio, W. Gao, C.M. Huang, L. Zhang, *Advanced Healthcare Materials*, 2 (2013) 1322-1328.
- [13] S.W. Jung, S. Thamphiwatana, L. Zhang, M. Obonyo, *PloS ONE*, 10 (2015).
- [14] S. Thamphiwatana, W. Gao, M. Obonyo, L. Zhang, *Proceedings of the National Academy of Sciences*, 111 (2014) 17600-17605.
- [15] J. Kabara, R. Vrable, M.L.K. Jie, *Lipids*, 12 (1977) 753-759.
- [16] J.J. Kabara, *Journal of the Society of Cosmetic Chemists*, 29 (1978) 733-741.
- [17] J.J. Kabara, *Journal of the American Oil Chemists' Society*, 61 (1984) 397-403.

- [18] J.J. Kabara, D.M. Swieczkowski, A.J. Conley, J.P. Truant, *Antimicrobial Agents and Chemotherapy*, 2 (1972) 23-28.
- [19] A.J. Conley, J.J. Kabara, *Antimicrobial Agents and Chemotherapy*, 4 (1973) 501-506.
- [20] J.J. Kabara, *Journal of the Society of Cosmetic Chemists*, 31 (1980) 1-10.
- [21] M. Howett, E. Neely, N. Christensen, B. Wigdahl, F. Krebs, D. Malamud, S. Patrick, M. Pickel, P. Welsh, C. Reed, *Antimicrobial Agents and Chemotherapy*, 43 (1999) 314-321.
- [22] M. Nazari, M. Kurdi, H. Heerklotz, *Biophysical Journal*, 102 (2012) 498-506.
- [23] I.M. Herzog, M. Fridman, *MedChemComm*, 5 (2014) 1014-1026.
- [24] G. Bergsson, J. Arnfinnsson, Ó. Steingrímsson, H. Thormar, *Journal of Pathology, Microbiology and Immunology*, 109 (2001) 670-678.
- [25] P. Mukerjee, K.J. Mysels, *Journal of the American Chemical Society*, 77 (1955) 2937-2943.
- [26] Â.S. Inácio, K.A. Mesquita, M. Baptista, J. Ramalho-Santos, W.L. Vaz, O.V. Vieira, *PloS ONE*, 6 (2011) e19850.
- [27] J.A. Jackman, N.-J. Cho, R.S. Duran, C.W. Frank, *Langmuir*, 26 (2009) 4103-4112.
- [28] A. Mechler, S. Praporski, K. Atmuri, M. Boland, F. Separovic, L.L. Martin, *Biophysical Journal*, 93 (2007) 3907-3916.
- [29] A. Mashaghi, S. Mashaghi, I. Reviakine, R.M. Heeren, V. Sandoghdar, M. Bonn, *Chemical Society Reviews*, 43 (2014) 887-900.
- [30] H.F. Chambers, F.R. DeLeo, *Nature Reviews Microbiology*, 7 (2009) 629-641.
- [31] M. Rodahl, F. Höök, A. Krozer, P. Brzezinski, B. Kasemo, *Review of Scientific Instruments*, 66 (1995) 3924-3930.
- [32] S.R. Tabaei, J.-H. Choi, G. Haw Zan, V.P. Zhdanov, N.-J. Cho, *Langmuir*, 30 (2014) 10363-10373.
- [33] J.A. Jackman, Z. Zhao, V.P. Zhdanov, C.W. Frank, N.-J. Cho, *Langmuir*, 30 (2014) 2152-2160.
- [34] R. Zoraghi, R.H. See, P. Axerio-Cilies, N.S. Kumar, H. Gong, A. Moreau, M. Hsing, S. Kaur, R.D. Swayze, L. Worrall, *Antimicrobial Agents and Chemotherapy*, 55 (2011) 2042-2053.

- [35] I. Wiegand, K. Hilpert, R.E. Hancock, *Nature Protocols*, 3 (2008) 163-175.
- [36] C. Keller, B. Kasemo, *Biophysical Journal*, 75 (1998) 1397-1402.
- [37] K. Glasmästar, C. Larsson, F. Höök, B. Kasemo, *Journal of Colloid and Interface Science*, 246 (2002) 40-47.
- [38] J. Kelsey, K. Bayles, B. Shafii, M. McGuire, *Lipids*, 41 (2006) 951-961.
- [39] S.R. Tabaei, J.A. Jackman, S.-O. Kim, V.P. Zhdanov, N.-J. Cho, *Langmuir*, 31 (2015) 3125-3134.
- [40] D. Thid, J.J. Benkoski, S. Svedhem, B. Kasemo, J. Gold, *Langmuir*, 23 (2007) 5878-5881.
- [41] S. Keller, H. Heerklotz, A. Blume, *Journal of the American Chemical Society*, 128 (2006) 1279-1286.
- [42] M. Staykova, M. Arroyo, M. Rahimi, H.A. Stone, *Physical review letters*, 110 (2013) 028101.
- [43] J.N. Israelachvili, D.J. Mitchell, B.W. Ninham, *Journal of the Chemical Society, Faraday Transactions 2: Molecular and Chemical Physics*, 72 (1976) 1525-1568.
- [44] E.N. Taylor, K.M. Kummer, D. Dyondi, T.J. Webster, R. Banerjee, *Nanoscale*, 6 (2014) 825-832.

Chapter 5

Correlating Membrane Morphological Responses with Micellar Aggregation Behavior of Capric Acid and Monocaprin*

In this chapter, the effects of the medium-chain saturated fatty acid, capric acid, and its monoglyceride derivative, monocaprin, on the morphological and fluidic properties of supported lipid bilayers (SLBs) were investigated. The critical micelle concentration (CMC) of each compound was determined by fluorescence spectroscopy measurements. At or above its CMC, capric acid induced the formation of elongated tubules protruding from the SLB, as determined by QCM-D and fluorescence microscopy experiments. By contrast, monocaprin induced the formation of elongated tubules or membrane buds below and above its CMC, respectively. FRAP experiments indicated that capric acid increased bilayer fluidity only above its CMC, whereas monocaprin increased bilayer fluidity both above and below its CMC. These findings are discussed within the context of the two compounds' structural properties, including net charge and hydrogen-bonding capacity. Collectively, the findings demonstrate that capric acid and monocaprin differentially affect morphological and fluidic properties of SLBs, and that the aggregation state of the compounds plays a critical role in modulating their interactions with phospholipid membranes.

*This chapter is published as B.K. Yoon, J.A. Jackman, M.C. Kim, T.N. Sut and N.J. Cho. Correlating Membrane Morphological Responses with Micellar Aggregation Behavior of Capric Acid and Monocaprin. *Langmuir* **33**, 2750–2759 (2017). DOI: 10.1021/acs.langmuir.6b03944.

5.1 Introduction

Single-chain lipid amphiphiles attract wide interest across fundamental and applied science, including fields such as molecular evolution, medicine, disinfectants and preservatives, renewable chemicals, food science, and biofuel production [1, 2]. While the lipid bilayers of biological membranes are typically composed of double-chain glycerophospholipid molecules, it is believed that biological life originated from the self-assembly of simpler building blocks such as short-chain fatty acids and monoglycerides into early cellular compartments [3-5]. Furthermore, single-chain amphiphiles have important biological functions (*e.g.*, antimicrobial activity, cell signaling), and are also known to interact with phospholipid bilayers, the latter of which has also inspired antimicrobial strategies aimed at destabilizing the lipid bilayers surrounding bacterial cells [6, 7]. Indeed, amidst the growing rise of antibiotic-resistant bacteria, free fatty acids and monoglycerides have emerged as promising antibacterial agents, both in the free form as preservatives and disinfectants as well as encapsulated within nanoscale drug delivery carriers as potential therapeutics [8]. From another perspective, the interaction of phospholipid membranes with free fatty acids is also important for the fermentative production of fatty acids for renewable chemical and biofuel production because high concentrations of fatty acids can damage bacterial cell membranes [9, 10].

For all these reasons, there is significant interest in understanding the interactions between single-chain lipid amphiphiles and phospholipid membranes [11]. To date, a wealth of knowledge has been obtained through antimicrobial studies which systematically investigated the influence of hydrocarbon chain properties (*e.g.*, chain length, degrees of unsaturation) and headgroup properties on *in vitro* bacterial growth [12-14]. These efforts revealed that single-chain lipid amphiphiles can damage bacterial cell membranes through either partial solubilization that hinders metabolic regulation (bacteriostatic growth inhibition) or membrane lysis (bactericidal cell death) [6]. In particular, medium-chain saturated fatty acids (between 6 and 18 carbons long) and corresponding monoglyceride derivatives were observed to have potent antibacterial effects, especially those with 10- and 12-carbon long chains [15]. Lauric acid (dodecanoic acid) demonstrated broad-spectrum inhibition of Gram-positive bacteria

while monolaurin (1-dodecanoyl-glycerol) was active at lower concentrations, albeit against a narrower range of bacteria [16, 17]. In marked contrast, monocaprin (1-decanoyl-glycerol) is more active against Gram-negative bacteria, especially those associated with foodborne illnesses [18, 19] and it also displays virucidal activity against enveloped viruses [20, 21]. Hence, while single-chain lipid amphiphiles broadly have membrane-disruptive activities, slight differences in the physiochemical properties (*e.g.*, chain length) can lead to dramatically different biological activities, necessitating experimental studies to delineate how these molecules differentially interact with lipid membranes.

In order to understand the morphological effects of treating bacteria, enveloped viruses, and other microorganisms with free fatty acids and monoglycerides, electron microscopy techniques have been employed for visualization of membrane damage and loss of cytoplasmic contents [22, 23]. Such experiments are conducted after treating the pathogen with high (5-10 mM) test agent concentrations followed by sample fixation [22]. Atomic force microscopy has also been employed in order to investigate the morphological effects of monoglyceride treatment against bacteria [24]. In terms of monitoring interaction kinetics, direct measurement of the interaction between single-chain lipid amphiphiles and phospholipid membranes has been achieved using solution-based liposome assays. Specifically, the insertion of fatty acids, namely oleic acid, linoleic acid, and capric acid as well as mixtures of oleic acid and oleate, into preformed, zwitterionic phospholipid vesicles was monitored by electron microscopy and dynamic light scattering [25, 26]. In general, it was observed that fatty acid monomers incorporate into the phospholipid vesicles and form mixed fatty acid-phospholipid vesicles, inducing partial solubilization that leads to vesicle growth and subsequent fission [27, 28]. The interaction of fatty acids with giant unilamellar vesicles (GUVs) has also been reported, and it was observed that the addition of oleic acid causes GUV growth followed by formation of membrane invaginations, evaginations, and budding, resulting in the formation of small daughter vesicles [29]. Furthermore, Mally *et al.* examined the partitioning of oleic acid into GUVs, and it was noted that partitioning, in this case, increases membrane strain and results in vesicle bursting upon reaching a critical strain in

the membrane [30]. Collectively, these studies highlight the potential of employing model membrane systems to study the dynamics of membrane-amphiphile interactions.

In particular, supported lipid bilayers (SLBs) on silicon oxide surfaces have enabled detailed mechanistic investigations by employing surface-sensitive measurement techniques [31, 32]. Thid *et al.* observed that the addition of a long-chain, polyunsaturated docosahexaenoic acid (DHA), at concentrations above its critical micelle concentration (CMC), induced morphological changes in the SLB, including the formation of worm-like, elongated lipid structures protruding from the bilayer [33]. Flynn *et al.* have further investigated the interactions between DHA and SLBs, and noted that the specific nature of these interactions can be complex and depend on many factors such as fatty acid concentration, phospholipid composition, and the types of ions in solution [34]. Recently, Yoon *et al.* observed that intercalation of either a medium-chain, saturated fatty acid (lauric acid) or a related anionic surfactant (sodium dodecyl sulfate) into SLBs induced membrane strain that caused the formation of protruding, worm-like tubule structures, whereas a nonionic monoglyceride (monolaurin) induced membrane budding, with the different behaviors tentatively attributed to the influence of molecular charge on membrane translocation [35]. In the same study, it was also observed that the free fatty acids and monoglycerides were appreciably more active at concentrations above their corresponding CMC values. Based on these previous investigations, there is evidence that free fatty acids and monoglycerides induce different kinds of membrane morphological responses, although the relationship between morphological responses and the aggregation behavior of single-chain lipid amphiphiles remains to be clarified and further extended to other biologically relevant molecules in each class.

The aim of the present study is to investigate the membrane morphological responses which capric acid and monocaprin induce in SLBs, and to establish a correlation between the micellar aggregation properties of these two compounds and the resulting effects on the morphological and fluidic properties of phospholipid bilayers. Fluorescence spectroscopy was employed in order to determine the CMC values of the two compounds in appropriate solution conditions. Quartz crystal microbalance-dissipation (QCM-D) experiments were conducted in order to monitor the concentration-dependent effect of the compounds on the mass and viscoelastic properties of

prefabricated SLBs, and fluorescence microscopy enabled the real-time observation of SLB morphological changes upon treatment with the two compounds. The effect of the compounds on lateral lipid diffusion within the SLB was also measured by fluorescence recovery after photobleaching (FRAP) analysis. Overall, the experimental studies reveal the influence of single-chain lipid amphiphile insertion on strain-induced membrane morphological responses as well as membrane fluidity, offering evidence that capric acid and monocaprin destabilize phospholipid membranes in different ways that depend on their corresponding molecular structure and aggregation state.

5.2 Materials and Methods

5.2.1 Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Capric acid, 1-pyrenecarboxaldehyde, and sodium dodecyl sulfate were procured from Sigma-Aldrich (St Louis, MO). Monocaprin was obtained from LGC Standards (Teddington, UK). Phosphate-buffered saline (PBS) was purchased from Gibco (Carlsbad, CA). All other reagents were obtained from Sigma-Aldrich. Milli-Q-treated water ($>18 \text{ M}\Omega\cdot\text{cm}$) (Millipore, Billerica, MA) was used for all solution preparation steps.

5.2.2 Preparation of Test Compound Solutions

Stock solutions of capric acid and monocaprin were first prepared by dissolving the appropriate quantity of test compound in ethanol to a final concentration of 400 mM. Then, the test compound solutions were prepared by typically diluting the stock solutions 100-fold with PBS, and the final concentration in the diluted solutions was 4 mM unless otherwise noted. In order to promote complete solubilization, the solutions were heated for 30 mins at 70 °C immediately before experiment. After heating, the solutions were cooled and then further diluted to the appropriate test concentration, typically in two-fold

dilution increments. All dry compounds were stored in a dark cabinet, and samples were prepared freshly on the day of experiment.

5.2.3 Fluorescence Spectroscopy

Experiments were performed with a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Australia) in order to determine the critical micelle concentration of the tested compounds. Measurements were conducted at room temperature (21 °C). The fluorescence emission spectrum of the probe, 1-pyrenecarboxaldehyde, in PBS was recorded upon excitation at 365.5 nm in the presence of increasing concentrations of the test compound. To prepare the sample, a stock solution of the probe was initially prepared in methanol at a final concentration of 5 mM. A certain amount of the probe stock was added to a glass vial and left to dry for 30 mins in order to fully evaporate the methanol. A PBS solution containing the appropriate amount of test compound was then added to hydrate the dried probe, followed by vortexing. The final concentration of the probe in the test solution was 0.1 μ M. All measurements for each sample were scanned six times and averaged.

5.2.4 Quartz Crystal Microbalance-Dissipation (QCM-D) Experiments

QCM-D experiments with a Q-Sense E4 instrument (Biolin Scientific, Stockholm, Sweden) were conducted in order to characterize the interaction between the test compounds and a prefabricated SLB platform. The QCM-D technique detects shifts in the resonance frequency (Δf) and energy dissipation (ΔD) of an oscillating, piezoelectric quartz crystal as a function of time, and these shifts relate to the acoustic mass and viscoelastic properties, respectively, of adsorbed biomolecules on the surface [36]. The sensor chips had a fundamental frequency of 5 MHz, and were coated with a sputter-coated, 50-nm-thick layer of silicon dioxide (model no. QSX 303, Biolin Scientific). The measurement data were collected at the third ($n=3$), fifth ($n=5$), and seventh ($n=7$) odd overtones using the QSoft software program (Biolin Scientific), and the data was normalized according to the overtone number. Data processing was performed in the

QTools (Biolin Scientific) and OriginPro 8.5 (OriginLab, Northampton, MA) software programs. All presented data was collected at the fifth overtone.

Before experiment, the chips were rinsed multiple times with water and ethanol, dried with nitrogen gas, and treated with oxygen plasma for 1 min using an Expanded Plasma Cleaner (model no. PDC-002, Harrick Plasma, Ithaca, NY). Initially, an SLB on the silicon dioxide surface was made by using the solvent-assisted lipid bilayer (SALB) method, as previously described [37, 38]. During experiments, the temperature in the measurement chamber was maintained at 25.0 ± 0.5 °C. A peristaltic pump (Reglo Digital, Ismatec, Glattbrugg, Switzerland) was used to inject liquid samples into the measurement chamber at a flow rate of 50 $\mu\text{L}/\text{min}$. Each measurement set was repeated at least twice.

5.2.5 Fluorescence Microscopy

Epifluorescence microscopy was performed in order to directly observe morphological changes in SLBs due to treatment with capric acid or monocaprin. Experiments were conducted with an Eclipse TI-U inverted optical microscope (Nikon, Japan) with a 60 $\times$ magnification (NA = 1.49) oil-immersion objective lens (Nikon), and images were collected with an iXon 512 pixel $\times$ 512 pixel EMCCD camera (Andor Technology, Northern Ireland). The pixel size was 0.267×0.267 μm^2 . A fiber-coupled mercury lamp (Intensilight C-HGFIE, Nikon) was used to illuminate fluorescently labeled phospholipids with a TRITC filter. SLBs were initially formed on glass slides attached to the microfluidic flow-through chamber (sticky slide VI 0.4, Ibidi, Germany) by using the vesicle fusion method (0.2 mg/mL extruded lipid vesicles with 72 nm average diameter). After formation, the SLB was rinsed with PBS, and then the test compound was introduced into the measurement chamber at a flow rate of 40 $\mu\text{L}/\text{min}$. Time-lapse micrographs were recorded every 5 s for a total duration of 30 min. The initial time, $t = 0$ s, was defined by when the test compound solution reached the channel inlets. The fluorescence intensity of each micrograph was normalized using a custom-written script for the Python(x,y) 2.7.5 software program.

5.2.6 Fluorescence Recovery After Photobleaching (FRAP) Measurements

FRAP measurements were carried out in order to monitor the lateral diffusivity of SLBs before and after treatment with the test compounds. A 20 μm diameter circular spot was photobleached for 5 s by using a 532 nm, 100 mW laser (Klasech Laser Technologies, Dortmund, Germany) and fluorescence micrographs were taken every 1 s for 90 s in total. Lateral diffusion coefficients were computed based on the Hankel transform method [39].

5.3 Results and Discussion

5.3.1 Determination of the Critical Micelle Concentration

The aggregation behavior of a surface-active compound is typically characterized by the critical micelle concentration (CMC), which is defined as the lowest bulk concentration of compound at which micellar aggregates begin to form. The CMC of an amphiphilic compound can vary widely depending on its environment (*e.g.*, solvent, ionic strength, temperature), and hence its CMC should be determined in the appropriate solution conditions. Therefore, the CMC values for capric acid and monicaprin in PBS were determined by measuring the fluorescence emission spectrum of pyrenecarboxaldehyde in the presence of increasing concentrations of test compound [40]. Specifically, the 1-pyrenecarboxaldehyde probe intercalates within micelles and therefore exhibits different emission properties in the presence and absence of micelles—a spectral feature which can be utilized for determining CMC values of test compounds [41]. In terms of data interpretation, the peak wavelength decreases in the presence of micelles and therefore the lowest concentration of test compound which causes a decrease in the peak wavelength is defined as the CMC value [42]. Literature reports also show agreement between CMC values obtained with the fluorescence probe method and surface tension measurements [42].

Before measuring the CMC values of capric acid and monicaprin, the measurement approach was first validated by characterizing the emission spectrum of the

probe and testing a reference sample, SDS, with known CMC values. Upon excitation at 365.5 nm, the emission spectrum of the probe in distilled water exhibited a peak wavelength around 472 nm and there was a 1-nm redshift (473 nm) in PBS, which is consistent with previously reported empirical trends between peak wavelength and the dielectric constant of the solvent [41, 43]. In control experiments, the CMC values of SDS in distilled water and PBS were determined to be 7 mM and 800 μ M, respectively. The value obtained in distilled water agrees well with literature values [44-46], while the result obtained in PBS demonstrates how high ionic strength decreases the CMC value due to charge shielding and other molecular binding interactions [47, 48].

Following this measurement approach, next the CMC values of capric acid and monocaprin in PBS were determined. **Figure 5.1** presents the peak wavelength of the fluorescence emission spectrum as a function of compound concentration. The determined CMC values for capric acid and monocaprin were 3.5 mM and 600 μ M, respectively. The lower CMC value of monocaprin can be explained by its nonionic

Figure 5.1 Determination of critical micelle concentration using the 1-pyrenecarboxaldehyde fluorescence probe. Peak wavelength is presented as a function of compound concentration in PBS solution for (A) capric acid and (B) monocaprin. The corresponding chemical structures of each compound are presented above each graph. Each data point is the average of six technical replicates ($n = 6$). The average and standard deviation (expressed as the error bars) for each data point are presented where applicable. The CMC value is defined as the highest test concentration at which no peak shift occurs.

character, and hence greater propensity to aggregate, as compared to anionic fatty acid molecules. Based on these values, QCM-D experiments were designed accordingly in order to investigate how the aggregation state of capric acid and monocaprin influences the mass and viscoelastic properties of prefabricated SLBs.

5.3.2 Effect on Mass and Viscoelastic Properties of Supported Lipid Bilayers

In order to investigate the interaction between the test compounds and SLBs, zwitterionic SLBs composed of the 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) phospholipid were initially prepared on silicon dioxide-coated, QCM-D sensor chips by the solvent-assisted lipid bilayer (SALB) technique [37, 38]. The single-component DOPC lipid composition was utilized because it provides a well-controlled model membrane to probe membrane morphological responses [33, 35]. As part of the SALB procedure, a baseline signal was first obtained in aqueous buffer solution (PBS), followed by exchange to isopropanol solution, incubation in 0.5 mg/mL DOPC lipid, and finally solvent-exchange back to PBS. The formation process was characterized by the QCM-D technique, and changes in the resonance frequency (Δf) and energy dissipation (ΔD) were recorded as a function of time. The Δf and ΔD shifts reflect the mass and viscoelastic properties of the lipid layer, respectively. SLB formation was successful, as judged by final Δf and ΔD shifts of -26 ± 2 Hz and $0.3 \pm 0.2 \times 10^{-6}$, respectively. These values are in agreement with those from literature reports [49], and indicate that the SLB is rigidly attached to the substrate with the expected areal mass density (460 ± 35 ng/cm²) as calculated by the Sauerbrey relationship which describes the relationship between the adsorbed mass and Δf shift for rigid thin films [50].

In order to determine the surface coverage of the SLB, 50 μ M bovine serum albumin (BSA) was added to bare and SLB-coated silicon dioxide surfaces. BSA adsorbs prodigiously onto silicon dioxide surfaces, but does not adsorb onto zwitterionic lipid bilayers [51]. Hence, this approach enables an estimation of bilayer surface coverage by taking into account that protein adsorption onto the bare substrate corresponds to 0% bilayer surface coverage ($\Delta f_{\text{control}}$) and assuming that reduced Δf shifts for BSA adsorption onto SLB-coated surfaces are proportional to the bilayer surface coverage. It

was determined that total BSA uptake was appreciably reduced on SLB-coated surfaces (-25 Hz *versus* -2 Hz or less for adsorbed protein layers on bare and SLB-coated surfaces, respectively), indicating the formation of high coverage (>93%) SLBs across the sensor surface (**Figure 5.2**).

Figure 5.2 QCM-D measurement of BSA protein adsorption onto bare and SLB-coated silicon dioxide surfaces. Changes in the QCM-D frequency signal were monitored as a function of time. The measurement baseline was recorded in PBS solution, and BSA protein was added starting at $t=5$ min (see arrow).

After SLB fabrication and the subsequent BSA blocking step, the measurement signals were stabilized for an additional 10 min before varying concentrations of capric acid or monocaprin were added to the SLB under continuous flow conditions. The resulting changes in mass and viscoelastic properties of the SLB platform, indicative of compound binding and membrane strain-dependent morphological responses, were tracked by monitoring the Δf and ΔD shifts. The SLBs were single-use and regenerated for each individual experiment. **Figures 5.3** and **5.4** present the corresponding QCM-D sensorgram results that were collected for the capric acid and monocaprin data sets. Note that the initial values of Δf and ΔD shifts at the normalized $t = 0$ min represent the values for an already formed SLB on the silicon dioxide surface.

5.3.2.1 Capric Acid

Figure 5.3 presents the effect of capric acid on the Δf and ΔD shifts as a function of capric acid concentration. Upon 8 mM capric acid treatment, there was a rapid decrease in Δf to around -103 Hz and increase in ΔD to 33×10^{-6} immediately after treatment (**Figure 5.3A**). After reaching inflection points, the measurement responses then began to reverse, with a rapid increase in Δf and decrease in ΔD to values of -16 Hz and 18×10^{-6} , respectively. The increase in Δf suggests that the capric acid treatment partially destabilized the SLB, along with gross morphological changes as evidenced by the large residual ΔD shift. Interestingly, when a buffer washing step was performed, a complex response in the Δf signal occurred with a net decrease to around -26 Hz whereas

Figure 5.3 QCM-D investigation of capric acid treatment on supported lipid bilayers. Δf (blue line with squares) and ΔD (red line with triangles) shifts as a function of time are presented for (A) 8 mM, (B) 4 mM, (C) 2 mM, and (D) 1 mM capric acid. The initial measurement values correspond to a supported lipid bilayer on the silicon dioxide surface. Capric acid was added at $t = 5$ min (arrow 1), and a washing step was performed (arrow 2) after the measurement signals stabilized.

the ΔD shift decreased to 0.2×10^{-6} . This behavior contrasts with previous examples of high concentrations of lauric acid treatment on SLBs, in which similar QCM-D signatures were observed for the fatty acid-SLB interaction albeit the washing step, in that case, led to more expected measurement responses, with a net Δf increase and a net ΔD decrease [35]. As with 8 mM capric acid, a similar activity profile was observed upon treatment with 4 mM capric acid (**Figure 5.3B**). The Δf signal decreased more gradually to -52 Hz before increasing and eventually stabilizing at -9 Hz. Concurrently, the ΔD signal followed the same trend and reached an inflection point at 18×10^{-6} before decreasing again and stabilizing at 6×10^{-6} . Again, the buffer washing step, in this case, showed a steep decrease in the Δf signal and increase in the ΔD signal to final values of -29 Hz and 0.5×10^{-6} , respectively. Upon treatment with capric acid at lower concentrations (2 mM and below), there were negligible changes in both the Δf and ΔD signals of less than -2 Hz and 1×10^{-6} , respectively (**Figure 5.3C, D**). Taken together, the QCM-D results indicate that capric acid is active when the capric acid concentration is greater than the CMC value (3.5 mM). At lower concentrations, capric acid is largely inactive against SLBs.

5.3.2.2 Monocaprin

Figure 5.4 presents the effect of monocaprin on the Δf and ΔD shifts as a function of monocaprin concentration. Upon treatment with 4 mM monocaprin, there was an immediate decrease in Δf to -147 Hz which occurs in parallel with a rapid increase in ΔD to 57×10^{-6} (**Figure 5.4A**). The Δf signal then decreased and eventually reached around -100 Hz before gradually increasing, while the ΔD signal reached around 50×10^{-6} before gradually decreasing. After a buffer washing step, there was a striking change in the measurement responses, yielding final Δf and ΔD shifts of -12 Hz and 1×10^{-6} , respectively. Upon treatment with 2 mM monocaprin, even larger shifts in both Δf and ΔD were observed reaching -270 Hz and 100×10^{-6} , respectively (**Figure 5.4B**). After the buffer washing step, the final Δf and ΔD shifts returned to values around -23 Hz and 0.5×10^{-6} , respectively. A similar trend in the measurement responses was observed with treatment at 1 mM monocaprin, but the maximum Δf and ΔD shifts were smaller, around

Figure 5.4 QCM-D investigation of monocaprin treatment on supported lipid bilayers. Δf (blue line with squares) and ΔD (red line with triangles) shifts as a function of time are presented for (A) 4 mM, (B) 2 mM, (C) 1 mM, (D) 500 μM , (E) 250 μM , (F) 125 μM , (G) 63 μM , (H) 31 μM , and (I) 16 μM monocaprin. The initial measurement values correspond to a supported lipid bilayer on the silicon dioxide surface. Monocaprin was added at $t = 5$ min (arrow 1), and a washing step was performed (arrow 2) after the measurement signals stabilized.

-145 Hz and 20×10^{-6} , respectively (**Figure 5.4C**).

When SLBs were treated with monocaprin at lower concentrations, there were appreciably smaller measurement responses. Upon treatment with 500 μM monocaprin, there was a more moderate decrease in Δf down to -42 Hz before gradually returning to -32 Hz. The corresponding ΔD shift increased to around 4×10^{-6} (**Figure 5.4D**). A buffer washing step led to a moderate decrease in the Δf signal and sharp drop in the ΔD signal to -24 Hz and 0.5×10^{-6} , respectively. Across the concentration range of 63 to 250 μM , treatment with monocaprin resulted in progressively smaller Δf and ΔD shifts with decreasing concentration, and the measurement responses occurred quickly before stabilizing (**Figure 5.4E-G**). In these cases, buffer washing led to final Δf and ΔD values of approximately -24 Hz and 0×10^{-6} , respectively. At lower monocaprin concentrations

(31 μM and below), treatment with monocaprin caused negligible Δf and ΔD shifts, indicating that monocaprin is inactive against SLBs in this low concentration range (**Figure 5.4H, I**). Collectively, at 1 mM and higher monocaprin concentrations, significant membrane morphological responses likely occurred based on the measurement responses. At intermediate concentrations between 63 and 250 μM , more moderate responses were observed, and there was negligible activity at lower concentrations. As the experimentally determined CMC value of monocaprin is 600 μM , the findings indicate that, in contrast to capric acid, monocaprin is active against SLBs at concentrations above *and* below its CMC value down to 63 μM . At the same time, the QCM-D measurement signatures suggest that the membrane morphological responses might differ depending on the specific monocaprin concentration, with the transition point occurring around the CMC value.

5.3.3 Observation of Morphological Changes in Supported Lipid Bilayers

To corroborate the QCM-D measurement signatures with specific membrane morphological responses, time-lapsed fluorescence microscopy experiments were performed that enabled direct microscopic observation of morphological changes in the SLB platform. An SLB composed of 99.5 mol% DOPC and 0.5 mol% 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) phospholipid was first fabricated on a silicon dioxide substrate within a microfluidic chamber. After bilayer formation, the test compound was introduced under continuous flow conditions and the resulting morphological changes were monitored with time-lapsed recording. The time point marked “ $t=0$ min” indicated when the solution containing the test compound reached the measurement chamber. Based on the QCM-D measurement results, capric acid and monocaprin were tested at two concentrations each, one above and one below the corresponding CMC value of each compound. The tested concentrations of capric acid were 4 and 1 mM, and the concentrations of monocaprin were 1 mM and 250 μM .

5.3.3.1 Capric acid

Figure 5.5 shows the time-lapsed sequence of morphological changes in an SLB upon treatment with 4 mM capric acid. **Figure 5.5A** presents the fluorescently labeled SLB, and the introduction of capric acid caused the formation of tubules protruding from the SLB (**Figure 5.5B**). While it appears that the tubules are brighter than the SLB, this effect is likely related to the tubules being out of the focal plane of the microscope lens [52]. The tubules varied widely in length (up to 100 μm), and exhibited parallel orientation to the SLB due to the flow direction in the microfluidic channel. The elongated tubule structures remained stable under continuous flow conditions for 30 min (**Figure 5.5C-E**). Of note, the flow condition is not necessary to induce membrane morphological responses in the SLB, but it does affect the orientation of the tubules relative to the SLB. When the flow was stopped, the elongated tubule structures

Figure 5.5 Microscopic observation of 4 mM capric acid-induced tubule formation on supported lipid bilayers. (A–F) Image snapshots at various time points depict nucleation sites from which tubules grow. $t = 0$ min corresponds to the introduction of 4 mM capric acid solution into the measurement chamber. The scale bar is 20 μm .

transitioned from a parallel orientation to a perpendicular orientation relative to the SLB (data not shown). Upon a buffer washing step, the tubules were removed and it was observed that the SLB remained underneath along with spots of bright fluorescence which appear to be nucleation sites for tubule growth (**Figure 5.5F**). The findings indicate that capric acid in the micellar form induces similar membrane morphological responses, namely the formation of elongated tubule structures, as caused by treatment with micelles of DHA [33] and lauric acid [35].

As presented in **Figure 5.6**, the effect of 1 mM capric acid on the SLB morphological properties was also evaluated in order to determine if low concentrations of capric acid below its CMC value would still be active against SLBs. In this case, only minor activity was observed, and there was a small number of tubules formed on the SLB surface and they remained stable (**Figure 5.6A-E**). Upon buffer washing, there was negligible change in the bilayer properties aside from removal of most tubes

Figure 5.6 Microscopic observation of 1 mM capric acid addition to supported lipid bilayers. (A–F) Image snapshots at various time points during after capric acid was added to the supported lipid bilayer. The growth of a small number of tubules was observed. $t = 0$ min corresponds to the introduction of 1 mM capric acid solution into the measurement chamber. The scale bar is 20 μm .

(**Figure 5.6F**). As the experiments were conducted under continuous flow conditions, the different morphological responses observed in the 1 mM and 4 mM capric acid cases did not vary due to the amount of mass uptake. Rather, the evidence supports that bulk concentration of capric acid is an important factor, and the compound is appreciably more active against SLBs in the micellar form than in the monomer form. The combination of QCM-D and fluorescence microscopy results further support that capric acid in the micellar form behaves similarly to other fatty acids, including DHA and lauric acid, and induces the formation of elongated tubule structures protruding from SLBs.

5.3.3.2 Monocaprin

Figure 5.7 shows the effect of treatment of 1 mM monocaprin on SLB morphological properties. Initially, a large number of small tubules were formed within the first 5 min (**Figure 5.7A**). While the experiments were conducted under continuous flow conditions, it was observed that many tubules did not follow the flow orientation and instead became entangled. As time progressed, the number of tubules decreased and there was an increasing number of bud-like structures that appeared within the SLB (**Figures 5.7C-E**). The buds are reminiscent of those induced by the interaction of monolaurin with SLBs, as previously reported [35]. After buffer washing, the buds and any residual tubules were removed and many defects within the SLB were apparent, as indicated by fluorophore-deficient regions (**Figure 5.7F**). Collectively, the results indicate that micellar aggregates of monocaprin induce membrane buds resulting from entangled tubules in similar fashion to monolaurin.

Figure 5.7 Microscopic observation of 1 mM monocaprin-induced bud protrusions on supported lipid bilayers. (A–F) Image snapshots at various time points depict nucleation sites from which entangled tubules grow and form buds. $t = 0$ min corresponds to the introduction of 1 mM monocaprin solution into the measurement chamber. The scale bar is 20 μm .

As shown in **Figure 5.8**, the effect of 250 μM monocaprin on SLB morphological properties was also investigated. In marked contrast to the capric acid case, monocaprin in the monomeric form still induced appreciable morphological changes in the SLB. Many tubules formed across the entire SLB surface and did *not* become entangled. Instead, the tubules behaved similarly to capric acid in its micellar form and nearly all the tubules formed in this case were oriented parallel to the SLB in the flow direction (**Figure 5.8A,B**). The tubules remained stable for 30 min, with only trace buds apparent (**Figure 5.8C-E**). Upon buffer washing, the majority of tubules were removed although a few tubules remained attached (**Figure 5.8F**). Importantly, many defects were also observed within the SLB after rinsing, however, they were appreciably smaller than those formed when the SLB was treated with monocaprin in the micellar form. This finding demonstrates that monocaprin in both the micellar and monomeric forms is active against

Figure 5.8 Microscopic observation of 250 μM monocaprin-induced tubule formation on supported lipid bilayers. (A–F) Image snapshots at various time points depict nucleation sites from which tubules grow. $t = 0$ min corresponds to the introduction of 250 μM monocaprin solution into the measurement chamber. The scale bar is 20 μm .

SLBs, and there is a distinct transition in the resulting membrane morphological responses from elongated tubules at lower concentrations (below CMC) to membrane buds at higher concentrations (above CMC). This finding clarifies previous observations that fatty acids are active only above the corresponding CMC due to the need for micelles to provide surfactant aggregates that facilitate membrane remodeling [33], whereas it was observed that monoglycerides, namely monocaprin, maintain some degree of activity down to 10-fold lower concentrations below the CMC.

From a broader perspective, the membrane morphological responses induced by fatty acids and monoglycerides appear to follow a spectrum of interactions whereby either elongated tubules or membrane buds can form depending on the specific conditions. As first described by Staykova *et al.*, confined lipid bilayers regulate stress through nucleation events that result in the formation of protrusions of different geometries; with

increasing membrane strain, it was noted that protrusions shift from budding cap to tubule morphologies [53]. Following this morphological explanation, a trend among the tested compounds was observed, with micellar aggregates of capric acid and monomeric monocaprin inducing greater membrane strain in SLBs than that caused by micellar aggregates of monocaprin. One important corollary observation is that monocaprin, but not capric acid, can form pore-like defects in SLBs. Monocaprin is a nonionic surfactant and, upon insertion into the lipid bilayer, can therefore translocate across leaflets on an appreciably quicker time scale than capric acid which is an anionic surfactant [54]. As such, it is reasonable to expect that monocaprin would exhibit detergent-like membrane solubilization due to translocation across the leaflets, whereas capric acid would not do so to an appreciable extent [55]. In addition to large-scale morphological changes, one remaining question concerns how the membrane insertion of capric acid and monocaprin affects lateral diffusion of phospholipids within the lipid bilayer.

5.3.4 Effect on Lateral Lipid Diffusion

To address this question, FRAP measurements were performed in order to determine the diffusion coefficient of a fluorescently labeled phospholipid probe within the SLB before and after treatment with capric acid and monocaprin at different concentrations. As shown in **Table 5.1**, treatment with 4 mM capric acid increased the diffusion coefficient from $2.90 \pm 0.05 \mu\text{m}^2/\text{s}$ to $3.51 \pm 0.03 \mu\text{m}^2/\text{s}$. By contrast, treatment with 1 mM capric acid had no effect as the diffusion coefficient remained similar before and after treatment. In contrast, treatment with monocaprin at both 1 mM and 250 μM concentrations led to an increase in SLB fluidity. Treatment with 1 mM monocaprin increased the diffusion coefficient from $3.04 \pm 0.25 \mu\text{m}^2/\text{s}$ to $3.77 \pm 0.23 \mu\text{m}^2/\text{s}$, while treatment with 250 μM monocaprin increased the diffusion coefficient from $3.11 \pm 0.07 \mu\text{m}^2/\text{s}$ to $3.64 \pm 0.22 \mu\text{m}^2/\text{s}$. The results demonstrate that both monomeric and micellar forms of monocaprin increase membrane fluidity whereas only micellar forms of capric acid increase membrane fluidity and monomeric capric acid has no effect.

Table 5.1 Summary of diffusion coefficients measured for supported lipid bilayers before and after treatment with capric acid or monocaprin, as determined by FRAP measurements.

Treatment	D ($\mu\text{m}^2/\text{s}$) Before Treatment	D ($\mu\text{m}^2/\text{s}$) After Treatment	% Change
4 mM Capric Acid^a	2.90 ± 0.05	3.51 ± 0.03	+21.0%
1 mM Capric Acid^b	2.62 ± 0.05	2.66 ± 0.03	+1.5%
1 mM Monocaprin^a	3.04 ± 0.25	3.77 ± 0.23	+24.0%
250 μM Monocaprin^b	3.11 ± 0.07	3.64 ± 0.22	+17.0%

^aConcentration is above the corresponding CMC value. ^bConcentration is below the corresponding CMC value.

In order to understand how these compounds increase membrane fluidity, it is important to recall previous work by Seu *et al.*, which demonstrated that insertion of lysophosphatidylcholine (another single-chain lipid amphiphile) into zwitterionic phospholipid SLBs also increased membrane fluidity [56]. By applying the Saffman-Delbrück model which relates lipid diffusion to membrane viscosity and height among other relevant parameters [57], it was explained that lysophosphatidylcholine increases membrane fluidity by decreasing the bilayer height along with weakening van der Waals interactions between molecules within the bilayer because it has only one hydrocarbon chain whereas glycerophospholipids have two hydrocarbon chains. As both capric acid and monocaprin have a single, medium-length hydrocarbon chain, similar arguments can be applied here in order to explain why these compounds increase membrane fluidity.

One last point concerns the observation that monocaprin, but not capric acid, exhibits activity in monomeric form. In general, monomeric single-chain amphiphiles are known to have short residence times within lipid bilayers [58]. Short residence times would hinder the accumulation of sufficient amounts of intercalated compound that are necessary to increase membrane strain and trigger morphological responses. Aside from the greater length of monocaprin which would likely increase residence time, another potentially important difference between capric acid and monocaprin is the capacity for hydrogen bonding with phospholipids in the lipid bilayer, which may also affect the residence time. Capric acid has a pK_a of 4.9 (Ref. [59]) and therefore its carboxylic acid functional group is deprotonated under the experimental conditions, hence the molecule

has limited potential for strong hydrogen bonding interactions with the phosphatidylcholine headgroup of DOPC molecules. By contrast, each monocaprin monomer has two –OH groups that can participate in hydrogen bonding interactions (*e.g.*, with the phosphate groups of glycerophospholipids [57]) so it may have more attractive interactions with one or more phospholipids, which would increase the residence time within the bilayer, enabling intercalation of a sufficient number of molecules at a given time to cause membrane strain and membrane morphological responses. Taken together, the experimental findings indicate that monocaprin is active against SLBs at concentrations above and below its CMC value, whereas capric acid is active only at concentrations above its CMC value. These findings are consistent with the molecular features of each compound and support that a spectrum of membrane morphological responses may occur depending on the physicochemical properties of the compound as well as its aggregation state.

As mentioned in the Introduction, there is broad interest in utilizing fatty acids and monoglycerides across a wide range of applications, and both capric acid and monocaprin have been explored. In this regard, most studies empirically report the membrane-disruptive behaviors (*e.g.*, antibacterial activity) of fatty acids and monoglycerides and there is scant discussion about the underlying interaction mechanisms or distinction between the activities of these two classes of single-chain lipid amphiphiles. These findings support that the activities of compounds in the two classes vary and can be comparatively understood by taking into account their different physicochemical properties. Such insights suggest that membrane-active antibacterial agents likely encompass a spectrum of interactions that might work synergistically. Furthermore, the dependence of the membrane-disruptive behavior on the monomeric *versus* micellar aggregation state is also reminiscent of past studies on other membrane-active surfactants [60] and antibiotics [61] and applying these insights might lead to the molecular design of amphiphilic compounds with improved therapeutic profiles. All of these features should be furthered explored in the context of different application possibilities in order to build on the fundamental insights obtained in this work.

5.4 Conclusion

In this work, the membrane morphological responses induced in SLBs arising from their interaction with capric acid and monocaprin, two single-chain amphiphiles of broad importance to fundamental and applied topics ranging from molecular evolution to anti-infective medicine, were investigated. Fluorescence spectroscopy experiments identified the CMC values for each compound, enabling the design of a comprehensive series of QCM-D, fluorescence microscopy, and FRAP experiments that evaluated the effect of these two compounds, in the monomeric and micellar aggregation states, on the morphological and fluidic properties of SLBs. The QCM-D experiments identified that capric acid is active against SLBs only above its CMC value, whereas monocaprin is active against SLBs both above and below its CMC value. Fluorescence microscopy experiments provided morphological evidence to support the QCM-D measurement results and further demonstrated that treatment with capric acid induced the formation of tubules only above its CMC value. By contrast, treatment with monocaprin induced the formation of tubules at concentrations below its CMC value *and* membrane buds at concentrations above its CMC value. Furthermore, monocaprin caused detergent-like membrane solubilization across the entire range of its membrane-active concentrations, whereas capric acid did not cause membrane solubilization even at sufficiently high concentrations (above CMC) to induce tubule formation. Coupled with the differential capacities of these two compounds to influence membrane fluidity as determined by the FRAP measurements, the findings identify that capric acid and monocaprin have variable effects on the morphological and fluidic properties of phospholipid membranes, and that the aggregation state of the compounds plays a critical role in modulating their interactions with phospholipid membranes. Key factors that deserve investigation in future work include establishing a molecular-level explanation for the different membrane activities of fatty acids versus monoglycerides as well as identifying opportunities to correlate these findings in model membrane systems with physiologically relevant functions such as antibacterial activity in biological membrane systems.

References

- [1] E. Boyle, J.B. German, J. Whelan, *Critical Reviews in Food Science & Nutrition*, 36 (1996) 785-805.
- [2] A.P. Desbois, *Recent Patents on Anti-Infective Drug Discovery*, 7 (2012) 111-122.
- [3] P.A. Monnard, D.W. Deamer, *The Anatomical Record*, 268 (2002) 196-207.
- [4] F. Olasagasti, M.-C. Maurel, D. Deamer, *Physico-chemical Interactions between Compartment-forming Lipids and Other Prebiotically Relevant Biomolecules*, EDP Sciences, (2014).
- [5] S.E. Maurer, D.W. Deamer, J.M. Boncella, P.-A. Monnard, *Astrobiology*, 9 (2009) 979-987.
- [6] A.P. Desbois, V.J. Smith, *Applied Microbiology and Biotechnology*, 85 (2010) 1629-1642.
- [7] H. Thormar, *Lipids and Essential Oils as Antimicrobial Agents*, John Wiley & Sons, (2011).
- [8] J.A. Jackman, B.K. Yoon, D. Li, N.-J. Cho, *Molecules*, 21 (2016) 305.
- [9] L.A. Royce, P. Liu, M.J. Stebbins, B.C. Hanson, L.R. Jarboe, *Applied Microbiology and Biotechnology*, 97 (2013) 8317-8327.
- [10] L.A. Royce, E. Boggess, Y. Fu, P. Liu, J.V. Shanks, J. Dickerson, L.R. Jarboe, *PloS one*, 9 (2014) e89580.
- [11] M. Ibarguren, D.J. López, P.V. Escribá, *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1838 (2014) 1518-1528.
- [12] J. Kabara, R. Vrable, M.L.K. Jie, *Lipids*, 12 (1977) 753-759.
- [13] J.J. Kabara, *Journal of the Society of Cosmetic Chemists*, 29 (1978) 733-741.
- [14] J.J. Kabara, *Journal of the American Oil Chemists' Society*, 61 (1984) 397-403.
- [15] G. Bergsson, Ó. Steingrímsson, H. Thormar, *International Journal of Antimicrobial Agents*, 20 (2002) 258-262.
- [16] J.J. Kabara, D.M. Swieczkowski, A.J. Conley, J.P. Truant, *Antimicrobial Agents and Chemotherapy*, 2 (1972) 23-28.
- [17] J.J. Kabara, *Journal of the Society of Cosmetic Chemists*, 31 (1980) 1-10.

- [18] A.J. Conley, J.J. Kabara, *Antimicrobial Agents and Chemotherapy*, 4 (1973) 501-506.
- [19] H. Thormar, H. Hilmarsson, G. Bergsson, *Applied and Environmental Microbiology*, 72 (2006) 522-526.
- [20] H. Thormar, C. Isaacs, H. Brown, M. Barshatzky, T. Pessolano, *Antimicrobial Agents and Chemotherapy*, 31 (1987) 27-31.
- [21] H. Thormar, C.E. Isaacs, K. Kim, H.R. Brown, *Annals of the New York Academy of Sciences*, 724 (1994) 465-471.
- [22] G. Bergsson, J. Arnfinnsson, Ó. Steingrímsson, H. Thormar, *Journal of Pathology, Microbiology and Immunology*, 109 (2001) 670-678.
- [23] G. Bergsson, J. Arnfinnsson, Ó. Steingrímsson, H. Thormar, *Antimicrobial Agents and Chemotherapy*, 45 (2001) 3209-3212.
- [24] M. Hyldgaard, D.S. Sutherland, M. Sundh, T. Mygind, R.L. Meyer, *Applied and Environmental Microbiology*, 78 (2012) 2957-2965.
- [25] S. Lonchin, P.L. Luisi, P. Walde, B.H. Robinson, *The Journal of Physical Chemistry B*, 103 (1999) 10910-10916.
- [26] M.L. Rogerson, B.H. Robinson, S. Bucak, P. Walde, *Colloids and Surfaces B: Biointerfaces*, 48 (2006) 24-34.
- [27] S. Chungcharoenwattana, H. Kashiwagi, M. Ueno, *Colloid and Polymer Science*, 283 (2005) 1180-1189.
- [28] S. Chungcharoenwattana, M. Ueno, *Chemical and Pharmaceutical Bulletin*, 53 (2005) 260-262.
- [29] P. Peterlin, V. Arrigler, K. Kogej, S. Svetina, P. Walde, *Chemistry and Physics of Lipids*, 159 (2009) 67-76.
- [30] M. Mally, P. Peterlin, S.a. Svetina, *The Journal of Physical Chemistry B*, 117 (2013) 12086-12094.
- [31] J.A. Jackman, N.-J. Cho, R.S. Duran, C.W. Frank, *Langmuir*, 26 (2009) 4103-4112.
- [32] A. Mechler, S. Praporski, K. Atmuri, M. Boland, F. Separovic, L.L. Martin, *Biophysical Journal*, 93 (2007) 3907-3916.
- [33] D. Thid, J.J. Benkoski, S. Svedhem, B. Kasemo, J. Gold, *Langmuir*, 23 (2007) 5878-5881.

- [34] K.R. Flynn, L.L. Martin, M.L. Ackland, A.A. Torriero, *Langmuir*, (2016).
- [35] B.K. Yoon, J.A. Jackman, M.C. Kim, N.-J. Cho, *Langmuir*, 31 (2015) 10223-10232.
- [36] M. Rodahl, F. Höök, A. Krozer, P. Brzezinski, B. Kasemo, *Review of Scientific Instruments*, 66 (1995) 3924-3930.
- [37] S.R. Tabaei, J.-H. Choi, G. Haw Zan, V.P. Zhdanov, N.-J. Cho, *Langmuir*, 30 (2014) 10363-10373.
- [38] S.R. Tabaei, J.A. Jackman, M. Kim, S. Yorulmaz, S. Vafaei, N.-J. Cho, *Journal of Visualized Experiments: JoVE*, (2015).
- [39] P. Jönsson, M.P. Jonsson, J.O. Tegenfeldt, F. Höök, *Biophysical Journal*, 95 (2008) 5334-5348.
- [40] K. Kalyanasundaram, J.K. Thomas, *Journal of the American Chemical Society*, 99 (1977) 2039-2044.
- [41] K. Kalyanasundaram, J.K. Thomas, *The Journal of Physical Chemistry*, 81 (1977) 2176-2180.
- [42] E. Goddard, N. Turro, P. Kuo, K. Ananthapadmanabhan, *Langmuir*, 1 (1985) 352-355.
- [43] N.J. Turro, T. Okubo, *The Journal of Physical Chemistry*, 86 (1982) 159-161.
- [44] I. Benito, M. Garcia, C. Monge, J. Saz, M. Marina, *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 125 (1997) 221-224.
- [45] E. Fuguet, C. Ràfols, M. Rosés, E. Bosch, *Analytica Chimica Acta*, 548 (2005) 95-100.
- [46] S.M. Bhairi, *Detergents: A Guide to the Properties and Uses of Detergents in Biological Systems*, Calbiochem-Novabiochem International, (2001).
- [47] L. Ambrosone, R. Ragone, *Journal of Colloid and Interface Science*, 205 (1998) 454-458.
- [48] P. Palladino, R. Ragone, *Langmuir*, 27 (2011) 14065-14070.
- [49] N.-J. Cho, C.W. Frank, B. Kasemo, F. Höök, *Nature Protocols*, 5 (2010) 1096-1106.
- [50] G. Sauerbrey, *Zeitschrift für Physik*, 155 (1959) 206-222.
- [51] K. Glasmästar, C. Larsson, F. Höök, B. Kasemo, *Journal of Colloid and Interface Science*, 246 (2002) 40-47.
- [52] J.C. Waters, *The Journal of Cell Biology*, 185 (2009) 1135-1148.

- [53] M. Staykova, M. Arroyo, M. Rahimi, H.A. Stone, Physical Review Letters, 110 (2013) 028101.
- [54] S. Keller, H. Heerklotz, A. Blume, Journal of the American Chemical Society, 128 (2006) 1279-1286.
- [55] H. Heerklotz, Quarterly Reviews of Biophysics, 41 (2008) 205-264.
- [56] K.J. Seu, L.R. Cambrea, R.M. Everly, J.S. Hovis, Biophysical Journal, 91 (2006) 3727-3735.
- [57] P. Saffman, M. Delbrück, Proceedings of the National Academy of Sciences, 72 (1975) 3111-3113.
- [58] J.N. Israelachvili, Intermolecular and Surface Forces: Second Edition, Academic Press, (1991).
- [59] P.H. Stahl, C.G. Wermuth, Handbook of Pharmaceutical Salts: Properties, Selection, and Use, John Wiley & Sons, (2008).
- [60] Y. Liu, S.L. Regen, Journal of the American Chemical Society, 115 (1993) 708-713.
- [61] K. Yamashita, V. Janout, E. Bernard, D. Armstrong, S.L. Regen, Journal of the American Chemical Society, 117 (1995) 6249-6253.

Chapter 6

Understanding How Sterols Regulate Membrane Remodeling in Supported Lipid Bilayers*

In this chapter, QCM-D experiments were conducted to investigate the membrane remodeling behaviors triggered by the addition of SDS, LA, and GML to SLB platforms composed of phospholipid and cholesterol mixtures. The SLB platforms were prepared by the SALB method in order to form cholesterol-rich SLBs with tunable cholesterol fractions (0-52 mol%). The addition of SDS or LA to fabricated SLBs induced tubule formation, and the extent of membrane remodeling was greater in SLBs with higher cholesterol fractions. In marked contrast, GML addition led to bud formation and the extent of membrane remodeling was lower in SLBs with higher cholesterol fractions. To explain these empirical observations, it is discussed how cholesterol influences the elastic (stiffness) and viscous (stress relaxation) properties of phospholipid/cholesterol lipid bilayers, as well as how the membrane translocation properties of single-chain lipid amphiphiles affect the corresponding membrane morphological responses. Collectively, the findings demonstrate that single-chain lipid amphiphiles induce highly specific membrane morphological responses across both simplified and complex model membranes, and cholesterol can promote or inhibit membrane remodeling by a variety of molecular mechanisms.

*This chapter is published as L.M. Kawakami¹, B.K. Yoon¹, J.A. Jackman, W. Knoll, P.S. Weiss, and N.J. Cho. Understanding How Sterols Regulate Membrane Remodeling in Supported Lipid Bilayers. *Langmuir* **33**, 14756–14765 (2017). DOI: 10.1021/acs.langmuir.7b03236.

(¹ denotes equal first authors)

6.1 Introduction

Single-chain lipid amphiphiles such as free fatty acids and monoglycerides play important roles in a wide range of biological and biotechnology applications ranging from molecular evolution to biofuels [1-3]. One of the most promising directions involves understanding how single-chain lipid amphiphiles interact with phospholipid membranes, giving rise to the targeted inactivation of pathogenic microbes such as viruses and bacteria. Previous studies have demonstrated that certain fatty acids and monoglycerides can lyse enveloped viruses [4, 5], including human immunodeficiency virus [6], herpes simplex virus [7], and respiratory syncytial virus [8]. Furthermore, some compounds within this class can disrupt the cellular membranes surrounding Gram-positive and Gram-negative bacteria, resulting in either bacterial cell death or growth inhibition [2, 9]. Within this scope, single-chain lipid amphiphiles that exhibit broad-spectrum antimicrobial activity against bacteria, viruses, and other microorganisms are termed “antimicrobial lipids”, and have been widely explored for several decades [10-14]. In the 1970’s and 1980’s, Kabara and colleagues conducted pioneering studies that investigated how the potency and scope of antimicrobial activity depend on the hydrocarbon chain length and number of degrees of unsaturation in antimicrobial lipids [15, 16]. Importantly, these efforts led to the discovery that medium-chain, saturated fatty acids and their monoglyceride derivatives – *i.e.*, lauric acid (LA) and glycerol monolaurate (GML) – exert particularly strong antimicrobial activity [17, 18] and these molecules have also been classified as Generally Recognized as Safe (GRAS) by the US Food and Drug Administration [2].

Expanding on these biological insights, there has been renewed interest in developing experimental strategies to characterize the interactions between single-chain lipid amphiphiles and phospholipid membranes. Such efforts are helping to provide a physicochemical explanation of why fatty acids and monoglycerides exhibit different spectra of antimicrobial activity. While direct observation of the interactions between antimicrobial lipids and biological membranes typically requires a fixed treatment period and sample fixation before imaging (*e.g.*, *via* scanning electron microscopy or atomic force microscopy), real-time monitoring of these interactions is possible using model

membrane platforms. To date, dynamic light scattering and electron microscopy techniques have been utilized to study how fatty acids can induce the aggregation of small vesicles, including subsequent fission and growth of individual vesicles [19, 20]. The shape transformation of giant unilamellar vesicles induced by oleic acid as well as the formation of new daughter vesicles has also been reported [21]. At the same time, while solution-based experimental systems provide insight into gross morphological changes, it is difficult to track molecular-level interactions at the nanoscale with such approaches, and surface-sensitive measurement platforms represent an alternative solution to address this measurement need.

In particular, supported lipid bilayers (SLBs) are simplified, two-dimensional phospholipid membranes that are commonly formed on silica-based surfaces and can be probed using surface-sensitive measurement techniques to monitor compound binding, membrane morphological responses, and changes in membrane fluidity. Using a single-component, zwitterionic phospholipid SLB platform, it was observed that the addition of docosahexaenoic acid (DHA) promoted the formation of elongated lipid protrusions as part of the membrane destabilization process [22]. Similar morphological responses were observed when DHA was added to SLBs of different phospholipid compositions and under different ionic conditions [23]. Importantly, it has also been possible to distinguish the membrane morphological responses induced by medium-chain saturated fatty acids and monoglycerides, as exemplified by studies involving LA and GML [24]. Like DHA, LA as well as sodium dodecyl sulfate (SDS) – an anionic detergent with a saturated 12-carbon-long hydrocarbon chain – induced the formation of elongated lipid protrusions, whereas neutral GML promoted the formation of membrane buds; the difference in morphological responses was attributed in part to how molecular charge influences membrane translocation and corresponding release of membrane strain [24]. Additional medium-chain saturated fatty acids and monoglycerides exhibited similar behavior against single-component SLBs, and a correlation between the molecular structure and micellar aggregation properties of the compounds and their differential effects on membrane morphology and fluidity was established [25]. On the other hand, biological membranes are more complex and composed of a milieu of different phospholipids and sterols, and therefore the morphological responses observed in simplified models should

be extended to more complex compositions. To this end, the formation of SLBs from *E. coli* and *S. cerevisiae* membrane extracts has been attempted, and the interactions of these SLB platforms with a monoglyceride were investigated [26]. However, it is challenging to prepare well-controlled SLBs with complex compositions, and a bottom-up approach to systematically investigate how specific membrane components affect membrane morphological responses would be advantageous for deciphering mechanistic details.

Aside from phospholipids, sterols and sterol-like compounds are ringed molecules that are found in biological membranes and help to regulate membrane fluidity, permeability, and rigidity [27, 28]. They also play important roles in membrane remodeling as part of biological processes such as endocytosis [29, 30]. In mammalian cells, cholesterol is the most prominent sterol and mammalian cell membranes contain as much as ~60 mol% cholesterol [31]. Unlike phospholipids, cholesterol molecules can rapidly translocate across bilayer leaflets and influence membrane properties [32, 33]. Likewise, bacterial cell membranes contain hopanoids that act as functional analogues of cholesterol [34]. While sterols and sterol-like hopanoids are prominent components in biological membranes, it is difficult to fabricate sterol-rich SLBs [35, 36] and solution-phase vesicles have been the main experimental system for studying the interactions between surfactants and phospholipid/cholesterol membrane compositions. For example, it has been observed that increasing amounts of cholesterol in vesicles impede membrane leakage and solubilization caused by SDS [37]. Extending these insights to uncover the influence of cholesterol on molecular-level interactions and corresponding membrane morphological responses is desirable, especially in the context of studying a broader range of membrane-active compounds like fatty acids and monoglycerides. Towards this goal, establishing cholesterol-rich SLB platforms would greatly advance measurement capabilities. While conventional fabrication methods are limited to preparing SLBs with no more than ~20 mol% cholesterol [36, 38, 39], it was recently reported that cholesterol-rich SLBs with up to ~57 mol% cholesterol can be formed using the solvent-assisted lipid bilayer (SALB) method [39, 40]. Such capabilities provide the basis for exploring how cholesterol, as a model sterol, influences membrane morphological responses induced by fatty acids and monoglycerides.

The goal of the present study was to investigate how cholesterol affects the

membrane morphological responses induced by representative fatty acids and monoglycerides, namely LA and GML; SDS was also tested. Zwitterionic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) SLB platforms were prepared with varying fractions of cholesterol (0-52 mol%) by the SALB formation method, and the addition of SDS, LA, and GML to prefabricated SLB platforms was tracked by quartz crystal microbalance-dissipation (QCM-D) measurements. The QCM-D technique offers a label-free measurement approach to monitor the changes in hydrodynamically-coupled mass and viscoelastic properties of an adsorbate, and therefore provides multiple signatures to characterize both SLB formation as well as subsequent membrane morphological responses, as previously established in multiple works [22, 24, 25]. Importantly, the QCM-D measurement signatures have been shown to correlate with the membrane morphological responses observed by fluorescence microscopy [24], and hence the QCM-D measurements provide insight into membrane remodeling behaviors, including the type and extent of membrane morphological response patterns (*i.e.*, tubule or bud formation). Following this measurement approach, it was examined how cholesterol influences membrane morphological responses induced by single-chain lipid amphiphiles, and discovered that the differential effects of cholesterol on regulating membrane remodeling depend on the elastic (stiffness) and viscous (stress relaxation) properties of phospholipid/cholesterol lipid bilayers.

6.2 Materials and Methods

6.2.1 Reagents

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Lauric acid, sodium dodecyl sulfate, and methyl-beta-cyclodextrin were obtained from Sigma-Aldrich (St Louis, MO). Glycerol monolaurate was purchased from Abcam (Cambridge, UK). The aqueous buffer solution was 10 mM Tris buffer solution [pH 7.5] with 150 mM NaCl. Phosphate-buffered saline (PBS) was purchased from Gibco (Carlsbad, CA). All other reagents were obtained from

Sigma-Aldrich. All solutions were prepared with Milli-Q-treated water ($>18 \text{ M}\Omega\cdot\text{cm}$) (Millipore, Billerica, MA). The dry compounds were stored in a dark cabinet.

6.2.2 Sample Preparation

DOPC lipid powder and cholesterol powder were dissolved in isopropanol at 10 mg/mL lipid concentration, mixed to the desired molar ratio, and then diluted to a 0.3 mg/mL total lipid concentration. Dissolved DOPC lipid powder and cholesterol powder were stored in -20°C fridge. Stock solutions of LA and GML were first prepared by dissolving the weighed compounds in ethanol to a final concentration of 200 mM. An aliquot from the ethanol solutions was diluted 100-fold with PBS to make 2 mM stock solutions and then further diluted in PBS as appropriate. The SDS test samples were prepared by dissolving the weighed compound in PBS (with 1% ethanol) to a 2 mM stock concentration and then further diluted in PBS as appropriate. To increase the solubility of all test samples, the solutions were heated in a 70°C water bath for 30 min. After heating, the solutions were cooled and then further diluted to the appropriate test concentration. All solutions were prepared on the same day as the experiments.

6.2.3 Quartz Crystal Microbalance-Dissipation (QCM-D)

QCM-D experiments with a Q-Sense E4 instrument (Q-Sense AB, Gothenburg, Sweden) were conducted in order to characterize the interactions between the test compounds and supported lipid bilayers with varying DOPC/cholesterol molar ratios. The QCM-D technique monitors changes in resonance frequency (Δf) and energy dissipation (ΔD) of an oscillating, piezoelectric quartz crystal sensor chip as a function of time, which reflect the acoustic mass and viscoelastic properties, respectively, of an adsorbate on the surface [41]. The sensor chip had a fundamental frequency of 5 MHz and a sputter-coated, 50-nm-thick layer of silicon dioxide (model no. QSX 303, Q-Sense AB). The experimental data were collected at the third ($n = 3$), fifth ($n = 5$), seventh ($n = 7$), ninth ($n = 9$), and eleventh ($n = 11$) odd overtones using the QSoft software program (Q-Sense AB), and the data were normalized according to the overtone number. Data

processing was performed in the QTools (Q-Sense AB) and OriginPro 8.5 (OriginLab, Northampton, MA) software packages. All presented data was collected at the seventh overtone. Before the experiment, the sensor chips were sequentially rinsed with water and ethanol, dried with nitrogen gas, and subjected to oxygen plasma treatment for 1 min with an Expanded Plasma Cleaner (model no. PDC-002, Harrick Plasma, Ithaca, NY). Initially, cholesterol-rich SLB on silicon dioxide surface was made by using the SALB technique [42]. During the experiments, the temperature in the measurement cell was maintained at 25.0 ± 0.5 °C. A peristaltic pump (Reglo Digital, Ismatec, Glattbrugg, Switzerland) was used to inject liquid samples into the measurement chamber at a flow rate of 50 μ L/min.

6.3 Results and Discussion

6.3.1 Formation of Cholesterol-Rich Supported Lipid Bilayers

Supported lipid bilayers on silicon dioxide substrates were prepared from mixtures of DOPC phospholipid and cholesterol by employing the solvent-assisted lipid bilayer (SALB) technique [39]. The QCM-D technique was employed to track the SLB formation process on silicon dioxide-coated sensor chips, and measures changes in resonance frequency (Δf) and energy dissipation (ΔD) that are related to an adsorbate's mass and viscoelastic properties, respectively [42]. For the QCM-D measurements, a baseline recording was first made in aqueous buffer solution, and then the solution was exchanged to water-miscible isopropanol solution. Afterwards, a 0.3 mg/mL mixture of DOPC and cholesterol in isopropanol – premixed to the desired molar ratio (0-52 mol% cholesterol input) – was deposited on the silicon dioxide surface, followed by solvent-exchange to aqueous buffer solution and this process resulted in SLB formation, as determined by the final Δf and ΔD shifts relative to the baseline recording.

The final QCM-D measurement values are presented in **Figure 6.1A,B**, and show a dependence on the molar fraction of cholesterol in the lipid mixture. For the DOPC phospholipid composition, the final Δf and ΔD shifts were -26 ± 1.5 Hz and $0.3 \pm 0.2 \times 10^{-6}$, respectively, which agree well with literature values for successful SLB formation [36, 43]. Similar values were obtained for DOPC/cholesterol SLBs with 17 mol%

Figure 6.1 QCM-D evaluation of cholesterol-rich SLB formation. QCM-D measurements were performed to characterize the SLB formation process. The final (A) Δf shifts and (B) ΔD shifts are reported after SLB formation was completed. The measurement shifts are relative to baseline recordings in equivalent aqueous buffer solution. The molar percentage (mol%) of cholesterol refers to the DOPC phospholipid/cholesterol mixture that was used in the SALB protocol. (C) QCM-D Δf shifts are reported as a function of time for cholesterol extraction from cholesterol-rich SLBs upon M β CD treatment. After SLB formation, the Δf shifts were normalized to baseline values at $t = 0$ min, and 1 mM M β CD was then added starting at $t = 5$ min. Positive Δf shifts indicate cholesterol extraction from the SLB platform. (D) Calculated molar percentage of cholesterol in SLB platform as a function of the input cholesterol fraction in the lipid mixture used in the SALB protocol. Mean and standard deviation (error bars) are reported from five or more technical replicates.

cholesterol, in which case the Δf and ΔD shifts were -26 ± 1.3 Hz and $0.6 \pm 0.2 \times 10^{-6}$, respectively. On the other hand, DOPC/cholesterol SLBs with 35 and 52 mol%

cholesterol fractions had appreciably larger measurement responses, and the final Δf and ΔD shifts were around -32 Hz and 1.3×10^{-6} , respectively. These variations in the QCM-D measurement responses as a function of cholesterol fraction are indicative of differences in membrane organization, prompting us to quantify the amount of cholesterol in the DOPC/cholesterol SLB platforms.

To determine the amount of cholesterol in the SLB platforms, the fabricated SLBs were treated with 1 mM methyl- β -cyclodextrin (M β CD), which can extract cholesterol from lipid membranes [44]. **Figure 6.1C** shows the normalized Δf shifts for fabricated SLBs after M β CD injection at $t = 5$ min. The normalized values at the initial time point correspond to successful SLB formation, and M β CD extraction of cholesterol from the SLBs led to positive Δf shifts, which indicates successful removal of cholesterol. Indeed, the corresponding kinetics exhibited monotonic behavior [44], and there was negligible measurement response when M β CD treatment was performed on DOPC SLBs. The magnitude of the Δf shifts increased according to the molar fraction of cholesterol used in the initial mixture. By applying the Sauerbrey model to quantify the mass of cholesterol removed and the mass of DOPC phospholipids remaining, the molar fraction of cholesterol in the SLB was calculated (**Figure 6.1D**) [39]. For 17 mol% cholesterol in the initial mixture, the resulting SLB had 12 ± 2 mol% cholesterol, and the SLBs formed from mixtures with 35 and 52 mol% cholesterol had 32 ± 2 and 48 ± 2 mol% cholesterol, respectively. Hence, the cholesterol fraction in the SLB platform could be tuned from ~10 to ~50 mol% cholesterol, and supports that the trend in QCM-D measurement responses relates to the effects of cholesterol fraction on membrane organization, as discussed below.

At relatively low molar fractions of cholesterol, mixtures of unsaturated phospholipids and cholesterol form a coexistence of liquid-disordered, fluid L_d -phases and liquid-ordered L_o -phases, the latter of which exhibits short-range orientational order and long-range translational disorder [45, 46]. Above a certain molar fraction of cholesterol (~20-30 mol%), phase coexistence no longer occurs and the mixture assumes the L_o -phase state [47]. This phase transition coincides with the condensing effect of cholesterol whereby the molecular density of the lipid mixture becomes greater as phospholipid and cholesterol form a stoichiometric complex at higher cholesterol

fractions [44]. Based on the amount of cholesterol in the SLB platforms as determined by MBCE extraction, the molecular area per lipid slightly decreases from $67 \text{ \AA}^2$ for a DOPC lipid membrane (0 mol% cholesterol input) to $60 \text{ \AA}^2$ for DOPC/cholesterol lipid membranes prepared from 17 mol% cholesterol input [48]. This small difference in molecular packing is consistent with the similar QCM-D measurement results obtained for these two cases. On the other hand, the molecular area per lipid decreases more appreciably to 45 and $42 \text{ \AA}^2$ for DOPC/cholesterol lipid membranes prepared from 35 and 52 mol% cholesterol inputs, respectively. Compared to the DOPC phospholipid case, the expected increase in molecular packing is around 33-37% for the higher cholesterol fraction cases, and this trend agrees well with the experimentally measured decrease in the QCM-D frequency shifts of around 19%. Indeed, as the magnitude of the QCM-D frequency shift is proportional to the adsorbed mass for rigid adlayers, the experimental findings support that the molecular density of SLBs with high cholesterol fractions is higher than conventional DOPC SLBs and SLBs with lower cholesterol fractions. Taken together, the data support that cholesterol-rich SLBs with < 20 mol% cholesterol likely coexist in the L_d and L_o phases, while cholesterol-rich SLBs with > 30 mol% cholesterol are in the L_o phase state.

6.3.2 Evaluation of Membrane Morphological Responses

After confirming the successful formation of DOPC/cholesterol SLBs, it was next investigated how cholesterol fraction influences membrane morphological responses induced by SDS, LA, and GML. The test compounds were added to prefabricated SLBs under continuous flow conditions, and QCM-D measurements were performed in order to track the corresponding membrane morphological responses, as established in previous studies [24, 25]. After SLB formation was complete, the QCM-D measurements were continued and the measurement times were normalized such that $t = 0$ min corresponds to the time after SLB formation followed by a stabilization period and $t = 5$ min indicates when compound addition to the SLB platform began under continuous flow conditions. Above their corresponding critical micelle concentration (CMC) values, the test compounds are known to induce membrane morphological responses and therefore

appropriate concentrations of each compound for evaluation were selected at 1 mM SDS, 2 mM LA, and 125 μ M GML. The QCM-D measurement responses are presented in **Figures 6.2-4**. Of note, at $t = 0$ min, the Δf and ΔD shifts correspond to SLB formation ($\Delta f_{\text{bilayer}}$ and $\Delta D_{\text{bilayer}}$) and the corresponding net Δf and ΔD shifts reported below are the $\Delta f_{\text{measured}} - \Delta f_{\text{bilayer}}$ and $\Delta D_{\text{measured}} - \Delta D_{\text{bilayer}}$ shifts, respectively. By reporting the net measurement shifts, it is possible to compare responses obtained on SLB platforms with different cholesterol fractions.

6.3.2.1 Sodium Dodecyl Sulfate

Figure 6.2 presents the effect of 1 mM SDS on the Δf and ΔD shifts as a function of cholesterol fraction in the SLB platform. SDS addition to 100 mol% DOPC SLBs exhibited two-step interaction kinetics, and there was an initially rapid decrease in the Δf shift to around -35 Hz and an increase in the ΔD shift to around 6×10^{-6} . These values corresponded to an inflection point that was followed by an increase in the Δf and ΔD shifts to -2 Hz and 0×10^{-6} , respectively (**Figure 6.2A**). After a buffer washing step, the final Δf and ΔD shifts were -1 Hz and 0×10^{-6} , respectively, and these final measurement responses are equivalent to a bare silicon dioxide sensor surface in buffer solution, indicating essentially complete removal of adsorbed lipid molecules. The interaction kinetics and final measurement result are consistent with SDS intercalation causing membrane strain that leads to tubule formation [24], resulting in membrane solubilization and SLB removal.

Interestingly, while cholesterol is known to increase membrane resistance to SDS-induced solubilization in suspended vesicle systems, similar two-step interaction kinetics were also observed for DOPC/cholesterol SLBs across all tested cases. In all cases, there was nearly complete membrane solubilization while the magnitude of the QCM-D measurement responses at the inflection point depended on the cholesterol fraction. For SLBs with 17 mol% cholesterol, the Δf and ΔD shifts reached their inflection points around -45 Hz and 7×10^{-6} , respectively (**Figure 6.2B**). Similarly, for SLBs with 35 and 52 mol% cholesterol, the Δf shifts at the inflection point were -50 and -54 Hz,

respectively (**Figure 6.2C,D**). The corresponding ΔD shifts were 10×10^{-6} and 12×10^{-6} , respectively.

Because the initial $\Delta f_{\text{bilayer}}$ and $\Delta D_{\text{bilayer}}$ shifts depended on the cholesterol fraction, the net Δf and ΔD shifts at the inflection point were also calculated for direct comparison across the different SLB platforms. The net Δf shifts were -9, -15, -19, and -21 Hz for 0, 17, 35, and 52 mol% cholesterol SLBs, respectively. The corresponding net ΔD shifts were 6, 7, 9, and 11×10^{-6} , respectively. These values indicate that the responses associated with tubule formation become larger with increasing cholesterol fraction in the SLB platform.

Figure 6.2 Effect of SDS treatment on mass and viscoelastic properties of cholesterol-rich SLBs. QCM-D measurements were performed, and representative Δf (blue squares) and ΔD (red triangles) shifts are reported as a function of time for DOPC phospholipid/cholesterol SLBs with (A) 0, (B) 17, (C) 35, and (D) 52 mol% cholesterol. The initial measurement values correspond to fabricated SLB platforms on the sensor surface. 1 mM SDS was added starting at $t = 5$ min (arrow 1), and a buffer washing step was then performed after the measurement signals stabilized (arrow 2).

6.3.2.2 Lauric Acid

Figure 6.3 presents the effect of 2 mM LA on the Δf and ΔD shifts as a function of cholesterol fraction in the SLB platform. As with SDS, LA addition to 100 mol% DOPC SLBs exhibited more subtle two-step interaction kinetics that also indicate tubule formation [24]. Upon LA addition, the Δf shift initially decreased to around -40 Hz with a concomitant increase in the ΔD shift to around 4×10^{-6} (**Figure 6.3A**). Then, the Δf shift began to increase until reaching around -35 Hz while the ΔD shift further increased to 6×10^{-6} . After a buffer washing step, the final Δf and ΔD shifts were around -19 Hz and 2×10^{-6} , respectively. These values indicate that adsorbed lipid mass remained on the sensor surface, supporting that LA induces membrane morphological responses without solubilizing the DOPC lipid bilayer.

With increasing cholesterol fraction, the interaction process exhibited more complex behavior and greater membrane solubilization was observed. For SLBs with 17 mol% cholesterol, the Δf and ΔD shifts reached their inflection points around -42 Hz and the corresponding ΔD shift was around 5×10^{-6} before there was a suddenly sharp increase in the ΔD shift to around 8×10^{-6} (**Figure 6.3B**). After a buffer washing step, the final Δf and ΔD shifts stabilized around -22 Hz and 3×10^{-6} , respectively. For SLBs with 35 mol% cholesterol, similar interaction kinetics were observed although the magnitude of the ΔD shift was more pronounced, reaching a maximum ΔD shift of 11×10^{-6} (**Figure 6.3C**). On the other hand, for SLBs with 52 mol% cholesterol, the Δf shift reached its inflection point around -49 Hz before increasing up to around -22 Hz while ΔD shift quickly rose and stabilized at around 14×10^{-6} throughout the interaction process (**Figure 6.3D**). Interestingly, upon buffer washing, the Δf shift rose to around -8 Hz and the ΔD shift decreased to around 3×10^{-6} . These measurement values indicate that LA induced the greatest extent of membrane solubilization for highly cholesterol-rich SLBs, suggesting that the stiffer membrane structures are more prone to destabilization.

For direct comparison, the net Δf and ΔD shifts at the inflection point for the different SLB platforms were reported. The net Δf shifts were -14, -14, -16, and -19 Hz for 0, 17, 35, and 52 mol% cholesterol SLBs, respectively. The corresponding net ΔD

Figure 6.3 Effect of LA treatment on mass and viscoelastic properties of cholesterol-rich SLBs. QCM-D measurements were performed, and representative Δf (blue squares) and ΔD (red triangles) shifts are reported as a function of time for DOPC phospholipid/cholesterol SLBs with (A) 0, (B) 17, (C) 35, and (D) 52 mol% cholesterol. The initial measurement values correspond to fabricated SLB platforms on the sensor surface. 2 mM LA was added starting at $t = 5$ min (arrow 1), and a buffer washing step was then performed after the measured signals stabilized (arrow 2).

shifts were 6 , 8 , 10 , and 13×10^{-6} , respectively. These values indicate that the measurement responses associated with tubule formation become larger with increasing cholesterol fraction in the SLB platform. This trend is particularly pronounced in the ΔD shift response, and mirrors the trends observed in the SDS case. As both SDS and LA are anionic molecules that induce tubule formation, the results suggest that the cholesterol fraction influences the morphological response to heightened membrane strain, likely stemming from how cholesterol affects the viscoelastic properties of lipid membranes.

6.3.2.3 Glycerol Monolaurate

Figure 6.4 presents the effect of 125 μM GML on the Δf and ΔD shifts as a function of cholesterol fraction in the SLB platform. GML addition to 100 mol% DOPC SLBs led to a gradual decrease in the Δf shift down to -112 Hz with a corresponding increase in the ΔD shift to 20×10^{-6} (**Figure 6.4A**). These values indicate that GML induces bud formation [24] and relates to the nonionic character of GML and its increased rate of membrane translocation across the two bilayer leaflets. After a buffer washing step, the final Δf and ΔD shifts were around -25 Hz and 1×10^{-6} , respectively, further indicating that GML does not cause membrane solubilization. Similar

Figure 6.4 Effect of GML treatment on mass and viscoelastic properties of cholesterol-rich SLBs. QCM-D measurements were performed, and representative Δf (blue squares) and ΔD (red triangles) shifts are reported as a function of time for DOPC phospholipid/cholesterol SLBs with (A) 0, (B) 17, (C) 35, and (D) 52 mol% cholesterol. The initial measurement values correspond to fabricated SLB platforms on the sensor surface. 125 μM GML was added starting at $t = 5$ min (arrow 1), and a buffer washing step was then performed after the measurement signals stabilized (arrow 2).

measurement trends were observed for cholesterol-rich SLBs, with marked decreases in the magnitudes of the Δf and ΔD shifts decreased at higher cholesterol fractions. For SLBs with 17 and 35 mol% cholesterol, the maximum Δf shifts were around -98 and -90 Hz, respectively, and the corresponding ΔD shifts were around 14 and 11×10^{-6} , respectively (**Figure 6.4B,C**). On the other hand, for SLBs with 52 mol% cholesterol, the maximum Δf shift was similar but the ΔD shift was only 8×10^{-6} (**Figure 6.4D**). In all cases, the final responses after the buffer washing step were roughly equivalent to the values obtained for SLBs, indicating that GML causes less membrane disruption to cholesterol-rich SLBs than SDS and LA.

As such, the net Δf shifts were -87, -69, -61, and -60 Hz for 0, 17, 35, and 52 mol% cholesterol SLBs, respectively. Likewise, the corresponding net ΔD shifts were 20, 14, 11, and 7×10^{-6} for 0, 17, 35, and 52 mol% cholesterol SLBs, respectively. This trend is opposite to that observed upon SDS and LA treatment in which cases the maximum ΔD shift increased for SLBs with greater cholesterol fractions. Hence, SDS and LA vs. GML had distinct effects on the membrane morphological response, including how cholesterol fraction influences the measured extent of remodeling behavior as well as the corresponding effects on membrane disruption as assessed post-treatment.

6.3.3 Influence of Cholesterol on Membrane Remodeling

The aforementioned results establish that the specific membrane morphological changes triggered by addition of SDS, LA, or GML to a SLB platform occur largely independent of the cholesterol fraction. This finding supports the general importance for lipid membranes to regulate stress passively through membrane remodeling and, in the case of confined, two-dimensional SLBs, such conditions give rise to membrane protrusions with distinct three-dimensional geometries [49, 50]. Indeed, the insertion of single-chain lipid amphiphiles and related compounds induces membrane strain (deformation) in SLBs, leading to nucleation events that promote membrane budding (*via* caps) or formation of elongated tubule structures [24, 25]. Nonionic compounds such as GML induce relatively low membrane strain leading to bud formation, while anionic compounds such as SDS and LA induce larger membrane strain that causes tubule

formation [24]. A key difference between these two cases is that nonionic GML molecules can quickly translocate across the two leaflets and equilibrate, whereas anionic molecules such as SDS and LA have slower rates of translocation and hence there is a concentration disequilibrium across the two leaflets, which contributes to larger membrane strains [9]. For all tested cholesterol fractions, the QCM-D data are consistent with these expected behaviors, with buds causing large shifts in the frequency and energy dissipation responses and tubules causing relatively small shifts in the frequency response and a large shift in the energy dissipation response. In both scenarios, the measurement responses arise from the formation of three-dimensional structures (buds or tubes) protruding from the surface, and larger measurement responses correlate with more significant morphological changes and membrane remodeling.

What is striking about the experimental data is that the effect of cholesterol fraction on the membrane morphological responses appears to depend on which particular single-chain lipid amphiphile was added. As presented in **Figure 6.5**, the maximum QCM-D measurement responses increased as the cholesterol fraction became greater for the SDS and LA cases, while the maximum QCM-D measurement responses decreased as the cholesterol fraction became greater for the GML case. In turn, this finding indicates that the extent of tubule formation was greater for SLBs with higher cholesterol fractions while the extent of membrane budding due to GML insertion decreased accordingly (**Figure A.1**). In other words, the extent of membrane remodeling depends not only on the cholesterol fraction but also which compound was added to the SLB platform.

To understand the physicochemical basis for this difference, let us consider the ways in which cholesterol affects lipid membrane properties. First, the addition of cholesterol is known to increase membrane stiffness in line with classical theories of elastic membranes [51, 52]. The two main contributing factors to the elastic energy of a curved lipid bilayer are the packing of individual lipid molecules (denoted by the spontaneous curvature) and the density disequilibrium of lipid molecules in the two leaflets arising from compression and expansion in the negatively and positively curved leaflets, respectively [53, 54]. Of note, equilibration of phospholipid densities across the two leaflets is slow because the energy barrier for phospholipid flip-flop is high. Treating

Figure 6.5 Trend in QCM-D measurement shifts for membrane remodeling behavior induced by different test compounds. Maximum Δf and ΔD shifts upon SDS, LA, or GML treatment are presented as a function of cholesterol fraction in the DOPC phospholipid/cholesterol SLB platforms. The net Δf and ΔD shifts are reported as $\Delta f_{\text{measured}} - \Delta f_{\text{bilayer}}$ and $\Delta D_{\text{measured}} - \Delta D_{\text{bilayer}}$ shifts, respectively, in order to normalize the magnitude of the measurement shifts across the different SLB platforms. Mean and standard deviation (error bars) are reported from three or more technical replicates.

a lipid bilayer as a purely elastic material, it would therefore be predicted that a cholesterol-rich bilayer would resist deformation more than a cholesterol-free bilayer. However, experimentally, it has been observed that cholesterol-rich membranes undergo greater deformation than pure phospholipid membranes [55, 56]. Such behavior has been explained by Szostak and colleagues by noting the propensity of cholesterol to flip-flop across the bilayer leaflets and aid stress relaxation by helping to equilibrate membrane density across the two leaflets [57]. As a result, cholesterol-rich membranes can deform to a greater extent than pure phospholipid membranes in response to stress, with transbilayer redistribution of cholesterol molecules helping to relax tension. This flip-flop behavior introduces a viscous component to phospholipid/cholesterol membranes, and deformation of complex, cholesterol-rich membranes exhibits a viscoelastic response [58]

that is influenced by the interplay of elastic (membrane stiffness) and viscous (cholesterol flip-flop) properties.

As mentioned above, the extent of membrane remodeling as a function of cholesterol fraction in the SLB depended on the particular compound triggering the membrane morphological response, and these differential trends can be understood by taking into account the physicochemical properties of each compound and how they relate to the elastic and viscous properties of the phospholipid/cholesterol SLB platform. For the GML case, the extent of membrane remodeling decreased with increasing cholesterol fraction, and this trend agrees with classical theories of membrane elasticity whereby deformation would decrease with increasing membrane stiffness (greater cholesterol fraction). This trend is consistent with the fact that GML can rapidly translocate across the bilayer leaflets and hence cholesterol flip-flop is likely a less important factor for stress relaxation. As such, the membrane morphological responses to GML addition appear to depend more strongly on the elastic properties of the SLB platform. By contrast, for the SDS and LA cases, the extent of membrane remodeling increased with increasing cholesterol fraction and this observation supports that the viscous properties of the SLB platform are the main contributing factor to influence the membrane morphological responses in these two cases. Indeed, there is a high energy barrier for anionic SDS and LA molecules to translocate across bilayer leaflets, giving rise to asymmetric strain and cholesterol flip-flop would therefore be an important factor in relaxing membrane tension. Taken together, the manner in which cholesterol affects membrane remodeling processes arising from SDS, LA, and GML addition to SLBs can be understood by taking into account how cholesterol influences the material properties of complex lipid bilayers as well as the physicochemical properties of the added compounds, especially molecular charge.

6.4 Conclusion

In this chapter, it was explored how cholesterol influences the membrane morphological responses that occur when single-chain lipid amphiphiles are added to SLB platforms. By employing the SALB method, it was possible to fabricate SLB

platforms with defined molar fractions of cholesterol across the fluid, liquid-disordered, and liquid-ordered phase states. It was discovered that the three tested compounds – SDS, LA, and GML – each induce specific and distinct membrane morphological responses in both pure phospholipid and phospholipid/cholesterol SLBs, indicating that the formation of three-dimensional protrusions is a generic mechanism to regulate stress in confined lipid bilayer systems. Interestingly, the presence of cholesterol strongly influenced the extent of membrane remodeling in a manner that depended on which compound was added. When SDS or LA was added to SLB platforms, elongated tubules formed and the extent of membrane remodeling was *greater* in SLBs with higher cholesterol fractions. In marked contrast, GML addition caused bud formation and the extent of membrane remodeling in this case was *lower* in SLBs with higher cholesterol fractions. As such, cholesterol either promoted or inhibited membrane remodeling and this multifaceted behavior could be understood by taking into account how cholesterol influences the elastic (stiffness) and viscous (stress relaxation) properties of phospholipid/cholesterol lipid bilayers, as well as how the membrane translocation properties of different single-chain lipid amphiphiles vary. Looking forward, such insights advance understanding of how cholesterol influences the viscoelastic properties of biological membranes and functions as a mediator of stress relaxation in lipid bilayers. In addition, the findings demonstrate that single-chain lipid amphiphiles such as free fatty acids and monoglycerides induce highly specific membrane morphological responses across both simplified and complex model membranes, motivating their continued exploration as antimicrobial candidates.

References

- [1] A. P Desbois, Recent Patents on Anti-Infective Drug Discovery, 7 (2012) 111-122.
- [2] A.P. Desbois, V.J. Smith, Applied Microbiology and Biotechnology, 85 (2010) 1629-1642.
- [3] J.A. Jackman, B.K. Yoon, D. Li, N.-J. Cho, Molecules, 21 (2016) 305.
- [4] J.A. Sands, D.D. Auperin, P.D. Landin, A. Reinhardt, S.P. Cadden, The Pharmacological Effect of Lipids (JJ Kabara, ed), American Oil Chemists' Society, Champaign, IL, (1978) 75-95.
- [5] W. Snipes, S. Person, G. Keller, W. Taylor, A. Keith, Antimicrobial Agents and Chemotherapy, 11 (1977) 98-104.
- [6] C.E. Isaacs, K. Kims, H. Thormar, Annals of the New York Academy of Sciences, 724 (1994) 457-464.
- [7] H. Hilmarsson, T. Kristmundsdóttir, H. Thormar, Journal of Pathology, Microbiology and Immunology, 113 (2005) 58-65.
- [8] H. Hilmarsson, B. Traustason, T. Kristmundsdóttir, H. Thormar, Archives of Virology, 152 (2007) 2225-2236.
- [9] H. Heerklotz, Quarterly Reviews of Biophysics, 41 (2008) 205-264.
- [10] H. Thormar, H. Hilmarsson, G. Bergsson, Microbial Pathogens and Strategies for Combating them: Science, Technology and Education. Méndez-Vilas A, editor. Formatex, 3 (2013) 1474-1488.
- [11] H. Thormar, G. Bergsson, E. Gunnarsson, G. Georgsson, M. Witvrouw, O. Steingrímsson, E. De Clercq, T. Kristmundsdóttir, Sexually Transmitted Infections, 75 (1999) 181-185.
- [12] H. Thormar, C.E. Isaacs, H.R. Brown, M.R. Barshatzky, T. Pessolano, Antimicrobial Agents and Chemotherapy, 31 (1987) 27-31.
- [13] Y. Li, Q. Xiang, Q. Zhang, Y. Huang, Z. Su, Peptides, 37 (2012) 207-215.
- [14] I.M. Banat, R.S. Makkar, S.S. Cameotra, Applied Microbiology and Biotechnology, 53 (2000) 495-508.
- [15] J.J. Kabara, Journal of the Society of Cosmetic Chemists, 29 (1978) 733-741.
- [16] J.J. Kabara, Journal of the American Oil Chemists' Society, 61 (1984) 397-403.

- [17] J.J. Kabara, D.M. Swieczkowski, A.J. Conley, J.P. Truant, *Antimicrobial Agents and Chemotherapy*, 2 (1972) 23-28.
- [18] A.J. Conley, J.J. Kabara, *Antimicrobial Agents and Chemotherapy*, 4 (1973) 501-506.
- [19] S. Lonchin, P.L. Luisi, P. Walde, B.H. Robinson, *The Journal of Physical Chemistry B*, 103 (1999) 10910-10916.
- [20] M.L. Rogerson, B.H. Robinson, S. Bucak, P. Walde, *Colloids and Surfaces B: Biointerfaces*, 48 (2006) 24-34.
- [21] P. Peterlin, V. Arrigler, K. Kogej, S. Svetina, P. Walde, *Chemistry and Physics of Lipids*, 159 (2009) 67-76.
- [22] D. Thid, J.J. Benkoski, S. Svedhem, B. Kasemo, J. Gold, *Langmuir*, 23 (2007) 5878-5881.
- [23] K.R. Flynn, L.L. Martin, M.L. Ackland, A.A. Torriero, *Langmuir*, 32 (2016) 11717-11727.
- [24] B.K. Yoon, J.A. Jackman, M.C. Kim, N.-J. Cho, *Langmuir*, 31 (2015) 10223-10232.
- [25] B.K. Yoon, J.A. Jackman, M.C. Kim, T.N. Sut, N.-J. Cho, *Langmuir*, 33 (2017) 2750-2759.
- [26] M. Hyldgaard, D.S. Sutherland, M. Sundh, T. Mygind, R.L. Meyer, *Applied and Environmental Microbiology*, 78 (2012) 2957-2965.
- [27] T.P. McMullen, R.N. Lewis, R.N. McElhaney, *Current Opinion in Colloid & Interface Science*, 8 (2004) 459-468.
- [28] H. Ohvo-Rekilä, B. Ramstedt, P. Leppimäki, J.P. Slotte, *Progress in Lipid Research*, 41 (2002) 66-97.
- [29] S.K. Rodal, G. Skretting, Ø. Garred, F. Vilhardt, B. Van Deurs, K. Sandvig, *Molecular Biology of the Cell*, 10 (1999) 961-974.
- [30] A. Subtil, I. Gaidarov, K. Kobylarz, M.A. Lampson, J.H. Keen, T.E. McGraw, *Proceedings of the National Academy of Sciences*, 96 (1999) 6775-6780.
- [31] Y. Lange, B. Ramos, *Journal of Biological Chemistry*, 258 (1983) 15130-15134.
- [32] W.D. Bennett, J.L. MacCallum, M.J. Hinner, S.J. Marrink, D.P. Tieleman, *Journal of the American Chemical Society*, 131 (2009) 12714-12720.

- [33] S. Jo, H. Rui, J.B. Lim, J.B. Klauda, W. Im, *The Journal of Physical Chemistry B*, 114 (2010) 13342-13348.
- [34] J.P. Sáenz, D. Grosser, A.S. Bradley, T.J. Lagny, O. Lavrynenko, M. Broda, K. Simons, *Proceedings of the National Academy of Sciences*, 112 (2015) 11971-11976.
- [35] L.X. Finegold, *Cholesterol in Membrane Models*, CRC Press, (1992).
- [36] M. Sundh, S. Svedhem, D.S. Sutherland, *Physical Chemistry Chemical Physics*, 12 (2010) 453-460.
- [37] M. Apel-Paz, G.F. Doncel, T.K. Vanderlick, *Langmuir*, 21 (2005) 9843-9849.
- [38] R.P. Richter, R. Bérat, A.R. Brisson, *Langmuir*, 22 (2006) 3497-3505.
- [39] S.R. Tabaei, J.A. Jackman, S.-O. Kim, B. Liedberg, W. Knoll, A.N. Parikh, N.-J. Cho, *Langmuir*, 30 (2014) 13345-13352.
- [40] S.R. Tabaei, J.A. Jackman, B. Liedberg, A.N. Parikh, N.-J. Cho, *Journal of the American Chemical Society*, 136 (2014) 16962-16965.
- [41] M. Rodahl, F. Höök, A. Krozer, P. Brzezinski, B. Kasemo, *Review of Scientific Instruments*, 66 (1995) 3924-3930.
- [42] G. Sauerbrey, *Zeitschrift für Physik*, 155 (1959) 206-222.
- [43] G.J. Hardy, R. Nayak, S.M. Alam, J.G. Shapter, F. Heinrich, S. Zauscher, *Journal of Materials Chemistry*, 22 (2012) 19506-19513.
- [44] H.M. McConnell, A. Radhakrishnan, *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1610 (2003) 159-173.
- [45] J.H. Ipsen, G. Karlström, O. Mourtsen, H. Wennerström, M. Zuckermann, *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 905 (1987) 162-172.
- [46] P.J. Quinn, C. Wolf, *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1788 (2009) 33-46.
- [47] D. Marsh, *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1798 (2010) 688-699.
- [48] M. Alwarawrah, J. Dai, J. Huang, *The Journal of Physical Chemistry B*, 114 (2010) 7516-7523.
- [49] M. Staykova, M. Arroyo, M. Rahimi, H.A. Stone, *Physical Review Letters*, 110 (2013) 028101.

- [50] A.J. Kosmalska, L. Casares, A. Elosegui-Artola, J.J. Thottacherry, R. Moreno-Vicente, V. González-Tarragó, M.Á. Del Pozo, S. Mayor, M. Arroyo, D. Navajas, *Nature Communications*, 6 (2015).
- [51] P.B. Canham, *Journal of Theoretical Biology*, 26 (1970) 61IN777-776IN881.
- [52] W. Helfrich, *Zeitschrift für Naturforschung C*, 28 (1973) 693-703.
- [53] L. Miao, U. Seifert, M. Wortis, H.-G. Döbereiner, *Physical Review E*, 49 (1994) 5389.
- [54] H.-G. Döbereiner, E. Evans, M. Kraus, U. Seifert, M. Wortis, *Physical Review E*, 55 (1997) 4458.
- [55] K. Bacia, P. Schwille, T. Kurzchalia, *Proceedings of the National Academy of Sciences of the United States of America*, 102 (2005) 3272-3277.
- [56] M. Yanagisawa, M. Imai, T. Taniguchi, *Physical Review Letters*, 100 (2008) 148102.
- [57] R. Bruckner, S. Mansy, A. Ricardo, L. Mahadevan, J. Szostak, *Biophysical Journal*, 97 (2009) 3113-3122.
- [58] S.-H. Wu, S. Sankhagowit, R. Biswas, S. Wu, M.L. Povinelli, N. Malmstadt, *Soft Matter*, 11 (2015) 7385-7391.

Chapter 7

Discussion and Future Work

This chapter outlines key findings and conclusions that were obtained in this thesis, and presents critical discussion of underlying scientific concepts that provide an analytical framework for characterizing antimicrobial lipids. Based on molecular properties of antimicrobial lipids that were identified to be physicochemical determinants of biophysical and biological activities, future directions are introduced that highlight how these fundamental insights can lead to the development of improved antibacterial strategies.

7.1 Discussion and Concluding Remarks

In this thesis, an integrated set of experimental approaches was developed to understand how antimicrobial lipids trigger membrane morphological responses in supported lipid bilayers and to correlate these concentration-dependent behaviors with the potency of antibacterial activity. Within this scope, particular attention was placed on medium-chain saturated fatty acids and corresponding monoglyceride derivatives, and a comprehensive analytical framework was established to i) characterize molecular self-assembly properties in bulk solution, including critical micelle concentration, ii) profile concentration-dependent interactions with supported lipid bilayers, iii) determine what type of membrane morphological responses are induced in supported lipid bilayers, iv) understand how membrane fluidity and integrity are affected by interactions with antimicrobial lipids, and v) evaluate the potency of antimicrobial lipids against *S. aureus* as a model bacterial pathogen. This framework was developed to test the overall hypothesis of this thesis that supported lipid bilayers can provide a model system to evaluate the mechanism of action and potency of antimicrobial lipids, and several key findings were made that demonstrate the utility of SLB platforms for characterizing antimicrobial lipids.

Before discussing the key findings of this thesis, it is important to emphasize the advantages of utilizing SLB platforms for studying the interactions between antimicrobial lipids and phospholipid membranes. First, SLB platforms provide a well-controlled, two-dimensional model membrane platform that can be integrated with a wide range of surface-sensitive measurement techniques. In this thesis, suitable experimental techniques were employed to characterize time-dependent interactions based on changes in mass and viscoelastic properties (to classify potency of antimicrobial lipids and initial profiling of membrane morphological responses), to observe the type of membrane morphological response induced by antimicrobial lipids (to provide direct observation of membrane morphological responses and visualize mechanistic steps), and to determine the effects of antimicrobial lipid treatment on membrane fluidity and integrity (to understand how single-chain lipid amphiphiles affect molecular organization of phospholipid molecules in the bilayer configuration). Collectively viewed, these measurement capabilities are

unparalleled for gaining molecular-level insights into how antimicrobial lipids interact with phospholipid membranes when compared to the experimental approaches available to study the interactions of antimicrobial lipids with solution-phase model membrane systems and bacterial cell membranes. Second, SLB platforms can be fabricated from a wide range of phospholipid and sterol compositions, providing a generalizable platform to investigate how membrane properties affect interactions with antimicrobial lipids. The versatility of SLB fabrication is particularly true with the recent development of the solvent-assisted lipid bilayer (SALB) method that enables high cholesterol fractions to be incorporated within high-quality SLBs. While the principal focus of this thesis was SLB platforms comprised of fluid-phase, single-component zwitterionic phospholipids, it was also demonstrated that more complex SLB platforms composed of phospholipids and cholesterol could be prepared with varying molar ratios. Based on detailed characterization analysis, it was further demonstrated that the resulting SLB platforms consisted of phospholipid-cholesterol membranes in different phase states that agreed with reported trends in membrane density. By combining the SLB platform with fluorescence spectroscopy measurements to determine the CMC values of antimicrobial lipids in bulk solution and MIC experiments to evaluate the antibacterial potency of antimicrobial lipids, several key findings were made in this this thesis that advance fundamental understanding about how antimicrobial lipids act against phospholipid membranes:

Spectrum of Membrane Morphological Responses

By employing QCM-D measurements and fluorescence microscopy, it was discovered that medium-chain saturated fatty acids and monoglycerides induce distinct patterns of membrane morphological responses in supported lipid bilayers. Specifically, it was identified that anionic fatty acids induce formation of elongated tubule structures protruding from the SLB, while nonionic monoglycerides induce the formation of spherical buds and caps. These findings were initially made with lauric acid and glycerol monolaurate, and later validated by capric acid and monocaprin. To explain these observations, it was explained how the different molecular properties of the two classes

of antimicrobial lipid, namely net electrical charge, affect the rate of membrane translocation across the SLB platform and hence influence the extent and spatial distribution of membrane strain in the bilayer. While antimicrobial lipids are broadly classified as a single class of molecule in the scientific literature, these findings provide the first initial evidence that two sub-classes of antimicrobial lipid – fatty acids and monoglycerides – differentially interact with phospholipid membranes, and hence the mechanisms of antibacterial activity are likely nuanced as well and deserving of further investigation in future work. Importantly, the findings in this thesis establish a fundamental physicochemical basis for comparing fatty acids and monoglycerides, shifting from empirical observations alone to deeper analysis.

Potency of Antimicrobial Lipids

In the scientific literature, it is often discussed how monoglycerides are more potent than fatty acids of equivalent chain length, however, the underlying reasons behind this trend were never discussed. In this thesis, it was identified that the interaction of antimicrobial lipids with SLB platforms strongly depends on the bulk lipid concentrations, and both fatty acids and monoglycerides were principally active only at or above their corresponding CMC values. Importantly, two additional experimental observations were made to correlate these biophysical observations with biological insights. First, it was determined that the CMC values of monoglycerides are approximately one order-of-magnitude lower than corresponding fatty acids. This difference was explained by taking into account the molecular properties of the two classes of antimicrobial lipids, especially net electrical charge. In general, nonionic surfactant-like molecules begin to form micelles at lower bulk concentrations than charged ones due to the lack of electrostatic repulsion in the former case. Hence, nonionic monoglycerides have lower CMC values than anionic fatty acid equivalents. Second, it was identified that the trend in CMC values correlated with the inhibitory concentrations of antimicrobial lipid that exerted antibacterial activity against *S. aureus* bacteria. In particular, antibacterial activity of the fatty acids and monoglycerides was observed around the corresponding CMC value and above, demonstrating that the physicochemical

properties of antimicrobial lipids are strongly linked to antibacterial potency. These findings are consistent with the empirical observations reported in the scientific literature, and provide the first physicochemical basis to explain the higher antibacterial potency of monoglycerides over fatty acids.

Effects on Membrane Fluidity and Integrity

Using the fluorescence recovery after photobleaching (FRAP) technique, it was further identified that antimicrobial lipids increase membrane fluidity within SLB platforms. The magnitude of the fluidity increase was similar for fatty acids and monoglycerides, and the results were in line with previous observations made for another class of single-chain lipid amphiphiles [1]. When antimicrobial lipids intercalate with the SLB platform, they disrupt packing interactions between double-chain phospholipid molecules, thereby decreasing the net interactions between molecules in the SLB platform and in turn increasing molecular diffusivity. While it has been suggested that antimicrobial lipids affect membrane fluidity in bacterial cell membranes [2], the FRAP measurements in this thesis are the first direct evidence of changes in membrane fluidity while previous biological measurements relied on indirect readouts such as ion leakage. One additional observation is that monoglycerides affected membrane fluidity at concentrations slightly below their CMC values, while fatty acids were active only above their CMC values. To explain this observation, it was further discussed how monoglycerides have greater net molecular lengths and hence individual molecules have longer residence times in the bilayer. In addition, monoglycerides have stronger interactions with phospholipid molecules due to hydrogen-bonding capacity, which also correlated with the propensity for monoglycerides to form microscopic pores in the SLB platforms while fatty acids had no such effect. These findings further reinforce that fatty acids and monoglycerides exhibit distinct interaction profiles with phospholipid membranes, emphasizing the connection between physicochemical properties and molecular self-assembly alongside biophysical and biological activities.

Influence of Sterols on Membrane Morphological Responses

While the preceding insights were obtained with SLB platforms composed of fluid-phase, single-component zwitterionic phospholipids, bacterial cell membranes and other cellular membranes often contain sterols that help to regulate membrane stress. Considering that antimicrobial lipids trigger membrane morphological responses, it was therefore pertinent to explore how sterols regulate these membrane morphological responses. By taking advantage of the SALB method, it was possible to investigate this question by fabricating SLB platforms composed of varying mixtures of phospholipid and cholesterol, and characterizing resulting membrane morphological responses induced by fatty acids and monoglycerides. In general, it was determined that antimicrobial lipids induce similar patterns of membrane morphological responses in phospholipid-only and cholesterol-containing SLB platforms. However, striking differences were observed in the manner in which cholesterol regulated the extent of membrane morphological responses, as evaluated by QCM-D measurements. For fatty acids, increasing cholesterol fractions in the SLB platform induced larger measurement responses indicative of more extensive tubule formation. In stark contrast, for monoglycerides, increasing cholesterol fractions in the SLB platform induced smaller measurement responses indicative of less extensive membrane budding. The distinct effects of cholesterol in either promoting or inhibiting membrane remodeling can be explained by taking into account how cholesterol affects the elastic (stiffness) and viscous (stress relaxation) properties of phospholipid membranes as well as considering the membrane translocation properties of fatty acid and monoglycerides. These findings were particularly important because they validated that the membrane morphological responses observed in single-component, zwitterionic phospholipid SLB platforms are representative of those occurring in more complex SLBs, thereby supporting that the SLB platform is a useful experimental system to characterize the potency of antimicrobial lipids. The experimental demonstration also demonstrates how SLB platforms can be fabricated in more sophisticated ways to answer outstanding biophysical questions and further reveal the distinct behaviors of fatty acids and monoglycerides acting against biologically relevant membrane compositions.

Taken together, the findings in this thesis demonstrate how different classes of antimicrobial lipids induce distinct membrane morphological responses in supported lipid bilayers, and how the concentration-dependent interaction profiles correlate not only with molecular-assembly properties of antimicrobial lipids in bulk solution but also with antibacterial activities against *S. aureus* bacteria. The work offers a comprehensive analytical framework to characterize antimicrobial lipids across biophysical and biological directions, and motivates numerous future research directions, three of which are discussed in the second half of this chapter.

7.2 Future Outlook

The findings reported in this thesis lay the groundwork to utilize SLB platforms for a wide range of mechanistic studies based on interfacing two-dimensional phospholipid membranes with other classes of surface-sensitive measurement techniques (*e.g.*, electrochemical sensors to detect antimicrobial lipid-induced pore formation) and membrane compositions, as well as studying different classes of antimicrobial lipids and mixtures thereof under varying environmental conditions towards evaluating potential antibacterial strategies. In this section, two examples are provided about how SLB platforms, and the broader analytical framework established in this thesis, can be utilized to study antimicrobial lipids across fundamental and application contexts.

7.2.1 Investigation of Glycerol Monolaurate Equivalent with Ether Backbone

As reported in this thesis, GML exhibits potent antibacterial activity against *S. aureus* bacteria [3-5], and this potency is driven by the length of the hydrocarbon chain (saturated, 12 carbon long chains are optimal) as well as by the nonionic headgroup properties that supports micellar aggregation at lower concentrations than fatty acids equivalents [6, 7]. While the molecular structure of GML has useful structural properties, there is one Achilles heel in its structure, namely the ester linkage connecting the lauric acid and glycerol building blocks. Indeed, *S. aureus* bacterial cells secrete an esterase enzyme that hydrolyzes ester linkages, rendering the conversion of GML into LA plus

free glycerol [8, 9]. As a consequence, the potency of hydrolyzed GML is much weaker and hence there is interest in developing stabilized versions of GML that are not susceptible to hydrolytic cleavage.

One potential solution involves utilizing 1-O-dodecyl-rac-glycerol (DDG), which is a conjugate of LA and glycerol with an ether linkage rather than an ester linkage (**Figure 7.1A**). As such, the compound is not susceptible to hydrolysis and it also has demonstrated antibacterial activity against *S. aureus* [10]. Interestingly, despite having nearly identical molecular structures, it is currently understood that GML and DDG have slightly different potencies and are believed to interact with bacterial cell membranes *via* distinct mechanisms [11]. To date, comparative investigations of DDG and GML have been strictly focused on biological activity profiling and extending the scope of investigations to biophysical approaches would improve understanding of how DDG and GML self-assemble and elicit antibacterial activity.

In reconnaissance work, the CMC properties of DDG and GML were investigated by fluorescence spectroscopy measurements (**Figure 7.1B**). While GML has a CMC value of 60 μM , the DDG has a lower CMC value of 40 μM , which is suggestive of more potent activity against phospholipid membranes. The difference in CMC values likely

Figure 7.1 (A) Chemical structure of glycerol monolaurate (GML) and dodecylglycerol (DDG) and (B) Determination of critical micelle concentration of DDG in PBS [1X, pH 7.2]. Each data point presented in the graph is the average value of six technical replicates ($n = 6$). Standard deviations (expressed as the error bars) are presented where applicable. The CMC value is defined as the highest test concentration before the first peak shift occurs.

arises from the greater polarity of ester linkages (more electron density) as compared to ether linkages, and the higher polarity would present a modest thermodynamic barrier to micellar aggregation at lower concentrations.

Fluorescence microscopy experiments have also been conducted to investigate how 31 μM DDG and GML interact with SLB platforms. As shown in **Figure 7.2**, GML induced small buds without membrane lysis while DDG induced more extensive tubule formation along with microscopic pore formation. Since both molecules have hydrogen-bonding capacities, the differences in membrane-lytic behaviors likely arise from concentration-dependent aggregation properties and the relationship between aggregation state and induction of membrane morphological responses. Overall, the preliminary data suggest that DDG is more membrane-lytic and exhibits greater potency than GML. Based on this groundwork, there is excellent potential for further investigating DDG and GML in a comparative fashion, including more comprehensive evaluation of biophysical interactions with SLB platforms and expanding the scope of investigation to evaluate the antibacterial activity and cytotoxicity of the two compounds.

Figure 7.2 Microscopic observation of 31 μ M GML- and DDG-induced membrane morphological responses on supported lipid bilayers. Image snapshots at various time points during after addition of (A) 31 μ M GML and (B) 31 μ M DDG to SLB platform are presented. $t = 0$ min corresponds to the introduction of antimicrobial lipid into the measurement chamber. The scale bars are 20 μ m.

7.2.2 Synergistic Mixtures of Fatty Acids and Monoglycerides

The primary objective of this thesis focused on characterizing individual types of antimicrobial lipids, namely fatty acids and monoglycerides, and it remains to be further understood how mixtures of fatty acids and monoglycerides induce membrane morphological activities and exhibit antibacterial activity. Indeed, mixtures of different surfactants are widely explored in various industries to improve functionality [12]. Inspired by research in this direction, some researchers have explored mixing antimicrobial lipids from different classes to achieve synergetic antibacterial effects. Sun *et al.* reported how mixing certain fatty acids and monoglycerides could yield additive antibacterial effects [13]. On the other hand, Batovska *et al.* reported instances where specific ratios of fatty acids and monoglycerides led to synergistic antibacterial effects [14]. To date, however, there has been no systematic investigation of how mixing different antimicrobial lipids influences molecular assembly or corresponding biophysical and biological activities.

As representative antimicrobial lipids with potent antibacterial activity, LA and GML represent excellent candidates for studying how mixtures of fatty acids and monoglycerides behave and at which concentrations mixed micelles begin to form. **Figure 7.3** presents the CMC values for GML/LA mixtures at different molar fractions. Depending on the molar fraction, the CMC value varied from 60 μM for 100 mol% GML to 950 μM for 100 mol% LA. With increasing GML molar fraction in the mixtures, the CMC value shifted from mirroring LA to approaching GML, and the values were lowered significantly even with small amount addition of GML, *e.g.*, 300 μM of CMC at 20 mol% of GML compared to that of LA, 950 μM .

In order to characterize the interaction strength between LA and GML within mixed micelles, the trend in CMC results as function of LA/GML ratio was modeled using a pseudo phase separation model [15-17]. The model relates the ideal mixed C_{mix}^* to the experimental CMC of the pure lipids, C_1 and C_2 , as well as the molar fraction of lipids 1 in the total mixed solute (α_1) through the following equation:

$$\frac{1}{C_{mix}^*} = \frac{\alpha_1}{C_1} + \frac{(1-\alpha_1)}{C_2} \quad [\text{Eq. 7.1}]$$

Figure 7.3 Determination of critical micelle concentration for GML/LA mixture. Peak wavelength is presented as a function of compound concentration in PBS solution for Different molar fraction of GML/LA mixture (GML:LA) as (A) 10:0, (B) 8:2, (C) 6:4, (D) 4:6, (E) 2:8, and (F) 0:10. Each data point presented in the graph is the average value of six technical replicates ($n = 6$). Standard deviations (expressed as the error bars) are presented where applicable. The CMC value is defined as the highest test concentration before the first peak shift occurs.

In cases of non-ideal mixing, the molar fraction of a particular lipid in the total mixture differs from its mole fraction in mixed micelles. By approximating solutions, the molar fraction of lipid 1 in the mixed micelle, termed x_1 , can be found iteratively through the following equation:

$$x_1^2 \ln \left(\frac{\alpha_1 C_{mix}}{x_1 C_1} \right) = (1 - x_1)^2 \ln \left(\frac{\alpha_2 C_{mix}}{(1 - x_1) C_2} \right) \quad [\text{Eq. 7.2}]$$

Using the x_1 obtained from the previous equation, the interaction parameter β can be obtained through the following equation:

$$\beta = \frac{\ln[(\alpha_1 C_{mix}/(x_1 C_1))]}{(1 - x)^2} \quad [\text{Eq. 7.3}]$$

Positive and negative β values indicate repulsion and attractive forces in a mixed micelle solution, respectively. In cases of ideal mixing, the β value should be close to 0. The average β value for the binary system of LA and GML was -0.8, which indicates that the

behavior of the binary surfactant system is nearly ideal. This observation can be explained by the weak interaction between anionic and nonionic molecular pairs, in which cause there is neither electrostatic attraction nor repulsion.

To further investigate how GML/LA mixtures induce concentration-dependent membrane morphological responses, QCM-D experiments were conducted by adding GML/LA mixtures at different molar ratios to prefabricated SLBs, as presented in **Figure 7.4**. While clear bud and tubule formation were observed at 100 mol% GML and ≥ 80 mol% LA, respectively, GML/LA mixtures at more intermediate molar ratios showed complex interaction profiles suggestive of dynamic membrane morphological responses that are driven by the competing types of membrane strain induced by fatty acids and monoglycerides. Hence, it was confirmed that GML/LA mixtures also induce membrane morphological responses and follow trends in CMC values.

Figure 7.4 QCM-D investigation of GML/LA mixture treatment on supported lipid bilayers.

Δf (blue line with circle) and ΔD (red line with triangles) shifts are presented as a function of time. Different molar fraction of GML/LA mixture (GML:LA) as (A) 10:0, (B) 8:2, (C) 6:4, (D) 4:6, (E) 6:4, and (F) 0:10 were treated above the corresponding CMC values. The baseline values at $t = 0$ min correspond to an SLB platform on the sensor surface. GML/LA mixture solution was added at $t = 5$ min (arrow 1), and a buffer washing step was performed (arrow 2) after the measurement signals stabilized.

This finding suggests the potential for antibacterial activity of GML/LA mixtures, and this possibility will need to be explored in future work. Looking forward, the

detected membrane morphological responses arising from GML/LA mixtures highlight the opportunities afforded by studying antimicrobial lipids with SLB platforms and employing comprehensive analytical frameworks that span biophysical and biological activity profiling.

References

- [1] K.J. Seu, L.R. Cambrea, R.M. Everly, J.S. Hovis, *Biophysical Journal*, 91 (2006) 3727-3735.
- [2] H. Alexandre, B. Mathieu, C. Charpentier, *Microbiology*, 142 (1996) 469-475.
- [3] J. Kabara, R. Vrable, M.L.K. Jie, *Lipids*, 12 (1977) 753-759.
- [4] J.J. Kabara, D.M. Swieczkowski, A.J. Conley, J.P. Truant, *Antimicrobial Agents and Chemotherapy*, 2 (1972) 23-28.
- [5] P.M. Schlievert, M.L. Peterson, *PloS one*, 7 (2012) e40350.
- [6] B.K. Yoon, J.A. Jackman, M.C. Kim, N.-J. Cho, *Langmuir*, 31 (2015) 10223-10232.
- [7] L.M. Kawakami, B.K. Yoon, J.A. Jackman, W. Knoll, P.S. Weiss, N.-J. Cho, *Langmuir*, (2017).
- [8] A. Ruzin, R.P. Novick, *Journal of Bacteriology*, 182 (2000) 2668-2671.
- [9] P. Schlievert, J.R. Deringer, M.H. Kim, S.J. Projan, R. Novick, *Antimicrobial Agents and Chemotherapy*, 36 (1992) 626-631.
- [10] P.J. McNamara, R.E. Syverson, K. Milligan-Myhre, O. Frolova, S. Schroeder, J. Kidder, T. Hoang, R.A. Proctor, *Antimicrobial Agents and Chemotherapy*, 53 (2009) 1898-1906.
- [11] Y.-C. Lin, P.M. Schlievert, M.J. Anderson, C.L. Fair, M.M. Schaefer, R. Muthyala, M.L. Peterson, *PloS one*, 4 (2009) e7499.
- [12] B. Kronberg, K. Holmberg, B. Lindman, *Surface Chemistry of Surfactants and Polymers*, John Wiley & Sons, (2014).
- [13] C.Q. Sun, C.J. O'Connor, A.M. Robertson, *FEMS Immunology & Medical Microbiology*, 36 (2003) 9-17.
- [14] D.I. Batovska, T. Todorova, V. Tsvetkova, H.M. Najdenski, *Polish Journal of Microbiology*, 58 (2009) 43-47.
- [15] R.F. Kamrath, E.I. Franses, *Industrial & Engineering Chemistry Fundamentals*, 22 (1983) 230-239.
- [16] K. Shinoda, E. Hutchinson, *The Journal of Physical Chemistry*, 66 (1962) 577-582.
- [17] P. Holland, D. Rubingh, *The Journal of Physical Chemistry*, 87 (1983) 1984-1990.

APPENDIX

Table A.1 Summary of experimental approaches to characterize antimicrobial lipids.

		Main Techniques	Technical Points
Biological Approaches	Growth Inhibition Assays	Minimum inhibitory concentration (MIC)	Determine the minimum concentration of antimicrobial lipids that inhibit bacterial growth. Evaluate capacity of a drug candidate by rapid screening of antibacterial activity against target bacteria. Does not provide direct information about the mechanism of antibacterial activity.
	Infectivity Assays	Minimum bactericidal concentration (MBC)	Determine the lowest concentration of antimicrobial lipid to kill a target bacterium. Evaluate if antibacterial activity is bacteriostatic or bactericidal. Does not provide direct information about interaction mechanism.
	Electron microscopy	TEM SEM	Enables direct observation of antibacterial effects of antimicrobial lipids against target bacterium. Visualizes morphological effects caused by treating target bacterium with antimicrobial lipids. Requires high concentration of antimicrobial lipids (≥ 5 mM) to treat target bacterium. Bacterial specimen must be fixed and prepared accordingly before imaging; real-time analysis is not possible.
Biophysical Approaches	Solution-Phase Liposomes (SUVs and LUVs)	Dynamic light scattering (DLS) Electron microscopy	Monitors interaction kinetics between antimicrobial lipids and phospholipid membranes by measuring changes in the size distribution of liposomes in bulk solution. Can utilize wide range of lipid compositions, including simple ones that are easy-to-prepare. Utilizes DLS as an ensemble-average measurement technique to determine in real-time the size and polydispersity of liposomes in bulk solution, and electron microscopy to visualize how antimicrobial lipids induce morphological changes in individual liposomes post-treatment.
	Giant Unilamellar Vesicle (GUV)	Phase-contrast and fluorescence microscopy	Visualizes morphological response induced by treatment with antimicrobial lipids in real-time. Can provide deep insights into morphological behaviors, including fluctuations and membrane fission/fusion.
	Supported Lipid Bilayers	QCM-D Fluorescence microscopy Fluorescence recovery after photobleaching (FRAP)	Monitors interaction kinetics between antimicrobial lipids and phospholipid membranes. Can be utilized with a wide range of surface-sensitive measurement techniques, allowing detailed investigation of binding mass, change in viscoelastic properties, and membrane fluidity.

Table A.2 Summary of critical micelle concentration (CMC) values for tested compounds used in this thesis.

Compound	CMC values	Ref.
SDS	260 μM^*	Inácio <i>et al.</i>
Lauric acid	1.5 mM*	Mukerjee <i>et al.</i>
GML	42 μM^*	Inácio <i>et al.</i>
Capric acid	3.5 mM**	Yoon <i>et al.</i>
Monocaprin	600 μM^{**}	Yoon <i>et al.</i>

* The CMC values were obtained from literature values and later reconfirmed in the Candidate's laboratory as part of later thesis work.

** The CMC values were experimentally measured for the first time as part of the work in this thesis.

Figure A.1 Schematic representation of cholesterol-rich membrane morphological responses to lauric acid and GML additions.