Design, characterization, and application of antiviral agent screening platform

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SINGAPORE

**DESIGN, CHARACTERIZATION, AND APPLICATION OF
ANTIVIRAL AGENT SCREENING PLATFORM**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2020

DESIGN, CHARACTERIZATION, AND APPLICATION OF ANTIVIRAL AGENT SCREENING PLATFORM

SOO HYUN PARK

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

2020

Statement of Originality

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

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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.

21 July 2020

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Professor Nam-Joon Cho

Authorship Attribution Statement

This thesis contains material from four paper(s) published in the following peer-reviewed journal(s) in which I am listed as an author.

Chapter 4 is published as S. Park, J.A. Jackman, and N.-J. Cho. Quantitative accounting of dye leakage and photobleaching in single lipid vesicle measurements: Implications for biomacromolecular interaction analysis. *Colloids and Surfaces B: Biointerfaces* **182**, 110338 (2019). DOI: 10.1016/j.colsurfb.2019.06.067.

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 co-designed the study with Professor Cho and performed all the laboratory work including sample preparation and fluorescence microscopy at the School of Materials Science and Engineering. I also analyzed all experimental data.
- Dr. Jackman advised on study design and data analysis.

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The contributions of the co-authors are as follows:

- Professor Cho and Professor Weiss provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Dr. Jackman, Professor Cho, and Professor Weiss.

- I designed the study, performed all the laboratory work including sample preparation and fluorescence microscopy at the School of Materials Science and Engineering. I also analyzed all experimental data.
- Dr. Jackman advised on study design and data analysis.
- Dr. Xu prepared poly(dimethylsiloxane) stamp and assisted with its characterization.

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- 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 and performed all the laboratory work including sample preparation, circular dichroism spectroscopy, fluorescence microscopy, and electrochemical impedance spectroscopy at the School of Materials Science and Engineering. I also analyzed all experimental data.
- Dr. Jackman advised on study design and data analysis.

21 Jul 2020

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Soo Hyun Park

Abstract

Emerging and reemerging viral pathogens are a major global health problem, and specific therapies and vaccines are unavailable for the vast majority of viral pathogens. Given the large number of circulating viruses, the development of effective antiviral strategies that work against multiple viruses is an urgent need. Conventional antiviral strategies inhibit the function of virus-specific components, which limits the range of susceptible viruses (“narrow-spectrum”), and drug-resistant virus strains often emerge quickly because these components are encoded within the error-prone viral genome. However, many medically important viruses possess an Achilles heel, namely a lipid envelope that surrounds virus particles and is a necessary structural component for viral infection. Strikingly, certain antiviral drugs, including specific amphipathic peptides, directly target the viral envelope and cause membrane destabilization, resulting in virus particle lysis and loss of infectivity. This novel antiviral strategy has several compelling features, including direct targeting of virus particles, efficacy against a wide range of enveloped viruses (“broad-spectrum”), and restrain the emergence of drug-resistant virus strains (because virus envelopes are obtained from the host cell membranes, not from the viral genome). However, mechanistic details about the membrane destabilization process remain to be understood, and tackling this problem requires innovative experimental strategies. Herein, the development of an antiviral peptide screening platform was reported that integrates soft matter design components together with biomaterial and surface functionalization strategies to facilitate highly parallel measurements tracking peptide-induced destabilization of nanoscale, virus-mimicking small unilamellar vesicles with tunable size and composition. The key design steps are presented, including tethering strategy, measurement concept, and data analysis, and important advantages of this single-particle tracking scheme are discussed vis-à-vis ensemble-averaging methods. To demonstrate the utility of this approach, the vesicle-rupture activity of an amphipathic, α -helical (AH) peptide, was investigated and further conducted a series of proof-of-concept experiments to optimize the screening platform. Additional experiments were conducted with complementary measurement tools in order

to validate the measurement readouts. In addition, a micropatterning strategy was applied to control the spatial position of tethered vesicles, thereby establishing a method to create highly ordered vesicle assemblies. Based on the successful development of this screening platform, two most promising antiviral peptide candidates were evaluated using this platform and other multiple techniques. By establishing structure-function relationships that correlate the amino acid sequences of peptides in this family with vesicle-rupture activity profiles, this work will improve our knowledge about the design principles behind membrane-active, antiviral peptides and lead to the identification of promising antiviral drug candidates to advance to biological evaluation. Looking forward, the measurement capabilities developed in this work will be broadly applicable to studying other classes of membrane-active agents, including small molecules, additional peptides, and enzymes, as well.

Lay Summary

Emerging and reemerging viral pathogens are a major global health problem, and specific therapies and vaccines are unavailable for the vast majority of viral pathogens many medically important viruses possess an Achilles heel, namely a lipid envelope that surrounds virus particles and is a necessary structural component for viral infection. Strikingly, certain antiviral drugs, including specific amphipathic peptides, directly target the viral envelope and cause membrane destabilization, resulting in virus particle lysis and loss of infectivity. This novel antiviral strategy is called Lipid Envelope Antiviral Disruption (LEAD). However, mechanistic details about the membrane destabilization process remain to be understood, and tackling this problem requires innovative experimental strategies. Herein, an antiviral agent-screening platform that integrates soft matter design components together with biomaterial and surface functionalization strategies to facilitate parallel measurements tracking peptide-induced destabilization of nanoscale, virus-mimicking small unilamellar vesicles with tunable size and composition is reported.

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Table of Contents

Abstract.....	i
Lay Summary	iii
Acknowledgements	v
Table of Contents.....	vii
Table Captions.....	xi
Figure Captions	xiii
Abbreviations.....	xxv
Chapter 1 Introduction	1
1.1 Problem Statement/Hypothesis	2
1.2 Objectives and Scope	5
1.3 Dissertation Overview	6
1.4 Findings and Outcomes	7
References	9
Chapter 2* Literature Review	13
2.1 Conventional Antiviral Treatment Strategy	14
2.1.1 Classification of Viruses	14
2.1.2 Classification of Established Antiviral Therapies	16
2.2 Lipid Envelope Antiviral Disruption (LEAD) Strategy	22
2.2.1 Classes of LEAD Antiviral Drugs	23
2.2.2 Amphipathic α -Helical (AH) Peptides.....	26
2.3 Experimental Approaches Using Model Membranes to Evaluate Antiviral Agents	
31	
2.3.1 Model Membranes to Mimic Biological Membranes.....	31

2.3.2 Vesicle Suspension and High-Throughput Screening.....	35
2.3.3 Surface-Sensitive Measurements.....	39
2.3.4 Tethered Lipid Vesicle Platform	45
References	51
Chapter 3* Experimental Methodology	63
3.1 Rationale for Selection.....	64
3.2 Materials	65
3.2.1. Amphipathic α -Helical (AH) Peptide	65
3.2.2. Lipid Reagents	66
3.2.3. Buffer Reagents.....	67
3.3 Preparation of Samples	67
3.3.1. Preparation of Peptide Solutions.....	67
3.3.2 Tethered Lipid Vesicle Preparation	68
3.3.3 Tethered Bilayer Lipid Membrane Preparation	73
3.4 Experimental Techniques.....	76
3.4.1 Peptide Concentration Measurement.....	76
3.4.2 Secondary Structure of Peptides	77
3.4.3 Dynamic Light Scattering (DLS).....	78
3.4.4 Fluorescence Microscopy	81
3.4.5 Electrochemical Impedance Spectroscopy (EIS)	88
References	91
Chapter 4* Quantitative Accounting in Single Lipid Vesicle Measurements.....	95
4.1 Introduction	96
4.2 Experimental Methods	97
4.2.1 Reagents	97

4.2.2 Vesicle Preparation	98
4.2.3 Tethered Vesicle Platform	99
4.2.4 Epifluorescence Microscopy	99
4.2.5 Image & Statistical Analysis	99
4.3 Results and Discussion	100
4.3.1 Quantification of Photobleaching Effect	100
4.3.2 Optimizing Membrane Composition	106
4.3.3 Quantitative Evaluation of a Membrane-Active Peptide	110
4.4 Conclusions	115
References	117
Chapter 5* Micropatterned Viral Membrane Clusters for Antiviral Drug	
Evaluation	121
5.1 Introduction	122
5.2 Experimental Methods	124
5.2.1 Reagents	124
5.2.2 Vesicle Preparation	125
5.2.3 PDMS Stamp Fabrication	125
5.2.4 Microcontact Printing (μCP)	126
5.2.5 Epifluorescence Microscopy	126
5.3 Results and Discussion	127
5.3.1 Design Rationale	127
5.3.2 Effect of Neutravidin Concentration on Tethered Density	131
5.3.3 Interaction between AH Peptide and Viral Membrane Clusters	136
5.4 Conclusions	139
References	139

Chapter 6* Comparing the Membrane Interaction Profiles of Two Antiviral Peptides: Insights into Structure-Function Relationship	143
6.1 Introduction	144
6.2 Experimental Methods	145
6.2.1 Reagents	145
6.2.2 Circular Dichroism (CD) Spectroscopy	146
6.2.3 Tethered Vesicle Platform	147
6.2.4 Vesicle Preparation	147
6.2.5 Epifluorescence Microscopy	148
6.2.6 Electrochemical Impedance Spectroscopy (EIS)	148
6.2.7 Structural Modeling.....	149
6.3 Results and Discussion.....	149
6.3.1 Secondary Structure Characterization	149
6.3.2 Real-Time Tracking of Peptide-Induced Vesicle Rupture	154
6.3.3 Peptide-Induced Membrane Leakage	157
6.3.4 Structural Modeling of Peptide Conformations	159
6.4 Conclusions	161
References	163
Chapter 7 Conclusion and Future Work	167
7.1 Conclusion and Discussion	168
7.2 Future Outlook	169
7.2.1 Optimization of the Lead Template Sequence	169
7.2.2 Preclinical Studies.....	175
References	180
List of Publications	181

Table Captions

Table 2.1 Clinically important enveloped viruses and currently available therapy with virus sizes.	15
Table 2.2 Comparison between AH and C5A peptide.....	30
Table 2.3 Previous literatures that studied AH peptide and various model platforms with various measurement techniques that will be discussed in the next section.	34
Table 2.4 Brief summary of experimental approaches using model membrane with pros and cons.	46
Table 6.1. Biophysical properties of AH and C5A peptides, including molecular weight (MW), isoelectric point (pI), net charge (Z), hydrophobicity (H), hydrophobic moment (μ H), and helical fraction (f H) are presented. The pI values were calculated from https://pepcalc.com/ , Z, H, and μ H were obtained from http://heliquet.ipmc.cnrs.fr/cgi-bin/ComputParamsV3.py , and f H was measured by CD spectroscopy analysis. For f H calculations, the measurements were conducted with 100 μ M peptide in Tris buffer and in a lipid membrane environment with a P:L ratio of 1:12.....	150
Table 7.1 Lead peptide sequences and their truncated or point mutated analogues that show different activities towards membrane interaction profiles as well as antiviral or antibacterial activities.	172

Figure Captions

Figure 1.1 Data refers to the specific cause of death as the percentage of total deaths. Note that the total death was estimated to be 56 million in 2017. The red shade in cause of death refers to infectious diseases and the number of deaths was calculated by percentage provided in the graph.3

Figure 2.1 Classification of established antiviral strategies. Replication inhibitors stop viral genome replication within infected host cells whereas entry inhibitors target existing virus particles before entry to the host cells or right after the release. The figure is adapted from Ref ⁴⁷.17

Figure 2.2 The LEAD strategy is to target and disrupt the viral membranes, which can be modeled by artificial lipid membrane and effective against various families of enveloped viruses.23

Figure 2.3 Molecular structure of RAFI with schematics of mechanism of action (A), cyclodextrin compounds (B), and CLR01 (C). The cartoon from panel (A) is adapted from Ref ⁹².25

Figure 2.4 Biological origin of the antiviral AH and C5A peptides, which correspond to the N-terminal amphipathic helix of the HCV NS5A protein.27

Figure 2.5 Helix net diagrams of AH, NH, and C5A peptides. The hydrophobic face is indicated with long continuous stretch of yellow shade in AH and C5A peptides whereas no sustained hydrophobic face was observed in NH peptide. The substituted or mutated amino acids compared to AH peptide are indicated as blue letters.28

Figure 2.6 Various simplified model membrane platforms to mimic complex biological membrane.33

Figure 2.7 Chemical structures and principle of fluorescence measurements that are commonly used in membrane permeabilization assays.37

Figure 2.8 The results of high-throughput orthogonal assay.¹⁵⁶ The y-axis refers to the first step, Tb³⁺ leakage, and the x-axis corresponds to the ratio of NBD-lipid quenching induced by accessible dithionite after equilibrium. Note that the lowest percentage (“No Peptide”) starts from ~55% as the outer leaflet of vesicles are quenched. (A) Alamethicin, melittin,

and LL37 were tested in two-step assay. The larger symbol sizes denote the higher total peptide concentrations. (B) 10,000 library compounds were tested to find out the most potent peptides derived from melittin.38

Figure 2.9 QCM-D analysis performed with SLB and lipid vesicle platforms to monitor interaction between AH peptide and membranes. (A) Left: Changes in resonance frequency (Δf) starting from the formation of SLB on silicon oxide. Note that AH peptide binds significantly higher within the biomembrane derived from Huh7 cell compared to artificial membrane with pure POPC. Right: Binding mass was derived from the Sauerbrey model.¹¹² (B) Left: Intact vesicles were adsorbed on the gold surface and AH peptide was injected ~50 min. The Δf and energy dissipation (ΔD) values of -26 Hz and less than 0.5×10^{-6} , respectively, indicated the formation of SLB.¹¹⁷ Right: The schematics of AH peptide-induced vesicle rupture kinetic is depicted (not drawn to scale). (C) The vesicle size dependency of AH peptide was tested by varying the extruded vesicle size distributions (as the nominal filter pore size is labeled). Complete rupture (last values indicating SLB) was only observed with less than 100 nm-filtered vesicles.¹¹⁵42

Figure 2.10 The interaction of AH peptide and 50-nm extruded lipid vesicles by varied peptide concentration. (A) QCM-D Δf and ΔD monitored during the interaction of AH peptide and adsorbed vesicles. The numbers, 1 and 2, indicate vesicle and peptide addition, respectively. (B) Optical mass changes obtained by ellipsometry measurements. (C) Summary values of ellipsometry shifts. Lipid, Peptide, Peptide + Lipid refer to the adsorbed vesicle layer, additional adsorbed peptide, and the final layer after the rupture process is complete, respectively.44

Figure 2.11 Various tethering strategies developed from Refs.^{174, 177, 180-183}. The most common substrate was glass and passivation method included supported lipid bilayer (SLB), BSA, and PLL-g-PEG. The tethering strategy included biotin-avidin complex, DNA-complementary DNA, and covalent bonding.47

Figure 2.12 The results obtained from single vesicle assay applied in membrane curvature sensing experiments to assess protein-lipid interaction.¹⁸⁷ (A) Surface plot of the F.I. obtained from vesicle and peptide, revealing higher F.I. from smaller vesicle (lower F.I.). (B) The protein density derived from F.I. versus vesicle diameter.48

- Figure 2.13** The results obtained from single vesicle assay applied in real-time monitoring of pore formation and membrane lysis.¹⁷⁷ (A) Schematic of the dual dye platform based on encapsulated calcein and membrane-embedded Rh-PE. (B) The real-time monitoring of F.I. during the injection of AH peptide. (C) F.I. profile of calcein based on the AH peptide concentration. (D) Histograms obtained from the release of calcein.49
- Figure 2.14** The comparison of unilamellar and multilamellar vesicles.¹⁸⁸ The single drop of signals observed from small (A) and large (B) unilamellar vesicles. The double (C) and triple (D) drops were observed from the multilamellar vesicles.50
- Figure 2.15** The combination of surface sensitive measurements and single vesicle platform to assess the interaction between anionic lipids, DOPG and DOPS, and α S.¹⁸⁹ (A) QCM-D measurements show the binding of α S more significantly to DOPS vesicles. (B) Scattering and fluorescence micrographs of tethered vesicles before (t_1) and after (t_2) the addition of α S. (C) The fraction of vesicles that are visible in scattering microscopy and the ratio between before and after the addition of α S.51
- Figure 3.1** Amino acid sequences of the tested antiviral peptides. Amino acids written in blue is conservative mutation whereas red letter represents non-conservative mutation. Additionally, amino acids shaded in grey, red, and blue indicate conserved hydrophobic, acidic, and basic residues, respectively.66
- Figure 3.2** Molecular structures of the tested lipid that are used for the thesis.67
- Figure 3.3** Avanti Mini-Extruder internal schematic (A) and fully assembled extruder apparatus (B).70
- Figure 3.4** An optical image of the assembled home-built microfluidic chamber indicating the inlet and outlet with a schematic showing the internal chamber.71
- Figure 3.5** Size exclusion chromatography (gel filtration) for lipid vesicle purification. (A) Principle of gel filtration. (B) Vesicle purification containing free calcein on a gel column.¹⁶ Vesicles are collected first (earlier fraction number) because the size of them are bigger than free calcein molecules.¹⁶72
- Figure 3.6** The schematic (A) and observation using epifluorescence microscopy in red (Rh-PE) and green (calcein) channel (B) of tethered vesicle platform.73
- Figure 3.7** Schematic illustrations of the tethered bilayer lipid membrane (tBLM) used in this thesis. (A) The sequential steps involved in the formation of tBLMs on the gold surface.

(B) The structure of the tether and spacer molecules with hydrophobic, hydrophilic, and disulfide residues shaded in grey, red, and yellow, respectively. (C) The formation of DMPC tBLMs using the tether and spacer molecules presented in (B). The figure is adapted from Ref. ²⁶.75

Figure 3.8 Size distribution of vesicles determined by DLS. The intensity-weighted DLS data shows the size distribution (reported as hydrodynamic diameter) for vesicle populations with different filter sizes of 50, 100 and 200 nm.81

Figure 3.9 Fluorescence-based microscopy techniques. From left, TIRF (total internal reflection fluorescence) microscopy, confocal microscopy, and epifluorescence microscopy are presented.....83

Figure 3.10 An overview scheme of the data analysis process of single vesicle platform by Python.84

Figure 3.11 (A) The comparison of manual subtraction of background intensity and Python correction. (B) Output after the second step of vesicle intensity extraction.85

Figure 3.12 Examples of data representation of single vesicle assay. (A) 100 nM and 1000 nM of AH peptide were tested to monitor pore formation (calcein) and membrane lysis (rhodamine). A single-step drop in fluorescence intensity is observed in both calcein and rhodamine. Insets are histograms of the time corresponding to the onset of pore formation or rupture obtained from >500 individual vesicles. (B) 10 nM of AH peptide was tested to evaluate vesicle size dependency. Left: Yellow solid and dot circle (A. and B.) track small-sized (72 nm) and big-sized (213 nm) vesicle respectively. Each shows significantly different kinetic that a small vesicle is ruptured completely whereas a big vesicle remains still with a long lag time. Right: Vesicle size was calculated based on the fluorescence intensity of each vesicle to study the relationship between vesicle size and the rupture of vesicles. Each data point represents an individual single vesicle.87

Figure 3.13 The equivalent circuit for modelling AC electrical impedance data. In this circuit, G_m represents the conduction of the membrane, which is primarily determined by membrane pore defects, C_m is the membrane capacitance.90

Figure 4.1 Schematic illustration of two-probe, highly parallel measurement platform for single vesicle analysis. (A) Molecular structures of the lipid and fluorophore components that are used for constructing the tethered vesicle platform. (B) Top: Schematic illustration

of tethered vesicle array for monitoring changes in membrane permeability (water-soluble calcein dye, green; encapsulated within lipid vesicles) and membrane rupture (rhodamine dye-labeled phospholipid, red; in a vesicle's lipid bilayer). Bottom: Time-lapsed imaging is performed, and changes in fluorescence properties of individual, sub-100 nm vesicles can be analyzed. Scale bars are 15 μm . (C) Representative full-view ($136.53 \mu\text{m} \times 136.53 \mu\text{m}$) microscopy images of tethered lipid vesicles. Top: encapsulated water-soluble calcein dye (green) markers. Bottom: Rhodamine dye-labeled phospholipids (red) markers in different excitation/emission channels. Scale bars are 20 μm 102

Figure 4.2 Time-dependent changes in calcein and rhodamine signals under different imaging interval conditions. (A) Schematic illustration of time-dependent changes in the two measurement signals (B) 3D fluorescence intensity profiles show time-resolved changes in calcein and rhodamine signals. Representative data are shown for a 1-min imaging interval. (C) Time-lapsed changes in the fluorescence intensity of the calcein signal for individual vesicles for different imaging intervals. (D) Statistical comparison of the final intensity of the calcein signal for individual DOPC lipid vesicles at the end of the 60-min measurement period. (E,F) Equivalent data for the rhodamine signal as shown in panels (C,D). *** $P < 0.001$ compared to other groups by two-tailed t-test. 105

Figure 4.3 Influence of DOPS phospholipid incorporation on calcein dye release. (A) Size distribution of vesicles determined by dynamic light scattering (DLS). The intensity-weighted DLS data shows the size distribution (reported as hydrodynamic diameter) for vesicle populations with different DOPS lipid fractions in the membrane composition. The control composition was 100 mol % DOPC. (B) Time-lapsed changes in the fluorescence intensity of the calcein signal for individual vesicles as a function of DOPS lipid fraction, with a 1-min imaging interval. (C) Quantification of the normalized fluorescence intensity of individual DOPC/DOPS lipid vesicles after 60 min. Each data point corresponds to a single vesicle. * $P < 0.05$, *** $P < 0.001$ compared to other groups by two-tailed t-test. (D) Change in fluorescence intensity for individual vesicles after 60-min measurement period as a function of vesicle diameter. The normalized calcein signal intensity, with 0-min baseline, is reported after a 60-min measurement period and plotted as a function of vesicle diameter..... 107

Figure 4.4 Influence of cholesterol incorporation on calcein dye release. (A) The DLS data shows the size distribution for vesicle populations with different cholesterol fractions in the membrane composition. The control composition was 7.5 mol % DOPS. (B) Time-lapsed changes in the fluorescence intensity of the calcein signal for individual vesicles for different cholesterol fractions, with a 1-min imaging interval. The DOPS lipid fraction was 7.5 mol %. (C) Quantification of the normalized fluorescence intensity of individual DOPC/DOPS/cholesterol lipid vesicles after 60 min. Each data point corresponds to a single vesicle. * $P < 0.05$, *** $P < 0.001$ compared to other groups by two-tailed t-test. (D) Change in fluorescence intensity for individual vesicles after 60-min measurement period as a function of vesicle diameter. The normalized calcein signal intensity, with 0-min baseline, is reported after a 60-min measurement period and plotted as a function of vesicle diameter..... 109

Figure 4.5 Single-vesicle tracking of AH peptide-mediated pore formation and vesicle rupture. (A) Schematic illustration of AH peptide-induced pore formation and vesicle rupture. (B) 3D fluorescence intensity profiles show time-resolved changes in calcein and rhodamine signals caused by AH peptide. The peptide concentration was 100 nM. Time-lapsed changes in the fluorescence intensity of the (C) calcein and (D) rhodamine signals for individual vesicles that were treated with different AH peptide concentrations, with a 0.4-min imaging interval. Histogram profiles of AH peptide-mediated pore formation and membrane lysis as a function of bulk peptide concentration. (E) Histograms of drop point in calcein signal for different bulk peptide concentrations. Each binned data point corresponds to an individual vesicle. (F) Histograms of the vesicle rupture (lag) time for different bulk peptide concentrations. Each binned data point corresponds to an individual vesicle. The experiments were conducted using 100 nM AH peptide and data depict mean $\pm$ s.d..... 111

Figure 4.6 Corrective measures for single-vesicle analysis of AH peptide-mediated pore formation. (A) Data normalization procedure to correct raw measurement data by removing the effects of dye photobleaching and nonspecific leakage. (B) Time-lapsed changes in the fluorescence intensity of the calcein signal for individual vesicles with different membrane compositions, with a 0.4-min imaging interval. Raw and corrected profiles refer to measurement data before and after normalization procedure is applied. The peptide

concentration was 100 nM. (C) Quantification of the initial rate of change in the calcein signal, without and with correction procedure, for individual vesicles with different membrane compositions. Each data point corresponds to a single vesicle. *** $P < 0.001$ for the corrected data set compared to the raw data set by two-tailed t-test.113

Figure 4.7 Corrective measures for single-vesicle analysis of AH peptide-mediated membrane lysis. (A) Time-lapsed changes in the fluorescence intensity of the rhodamine signal for individual vesicles with different membrane compositions, with a 0.4-min imaging interval. Raw and corrected profiles refer to measurement data before and after the normalization procedure is applied. The peptide concentration was 100 nM. (B) Quantification of the lag time of the rhodamine signal, without and with correction procedure, for individual vesicles. Each data point corresponds to a single vesicle.115

Figure 5.1 Schematic illustration of the experimental strategy. Micropatterned viral membrane clusters mimic the architecture of viral particle clusters, which often display heightened functionality compared to individual particles. The clusters consist of enveloped virus-mimicking vesicles akin to viral particle aggregates.123

Figure 5.2 Design of the viral membrane cluster platform. (A) Schematic illustration of tethering individual vesicles. (B) Fluorescence microscope images of individually tethered vesicles (~100 nm diameter), as indicated by calcein and rhodamine signals. All scale bars are 10 μm128

Figure 5.3 Microcontact printing. (A) Schematic illustration describing microcontact printing (μCP) for location-specific patterning of neutravidin protein molecules. (B) Atomic force microscope (AFM) image of a polydimethylsiloxane (PDMS) stamp with pyramidal stamp pattern. (C) Height profile from AFM scan corresponding to PDMS stamp shown in (B). (D) Fluorescence microscope images of FITC-labeled neutravidin on a bare glass substrate. A scale bar is 10 μm129

Figure 5.4 (A) Schematic illustration describing μCP strategy to form micropatterned viral membrane clusters. (B) Fluorescence microscope images of patterned vesicles on a PLL-g-PEG/PLL-g-PEG-biotin functionalized glass surface, as indicated by calcein and rhodamine signals. All scale bars are 10 μm131

Figure 5.5. Fluorescent microscope images comparing immobilization of tethered vesicles in single-vesicles and clustered vesicle arrangements. The calcein and rhodamine signals

are presented for each experimental set. During fabrication, the bulk neutravidin concentration was varied from 0 to 100 $\mu\text{g/ml}$ in order to control the density of tethered vesicles. The number of tethered vesicles or clusters identified by the calcein or rhodamine signals are reported in the upper right image and are based on imaging in ten different fields of view. For the non-patterned platform, it was not possible to quantify the number of individual vesicles for the 100 $\mu\text{g/ml}$ neutravidin case. All scale bars are 20 μm 134

Figure 5.6 Fluorescence intensity quantification of liposome clusters. Particle counting procedure with full image view ($137 \times 137 \mu\text{m}^2$) of (A) control experiment (0 $\mu\text{g/ml}$ neutravidin), (B) 0.1 $\mu\text{g/ml}$, (C) 1 $\mu\text{g/ml}$, (D) 10 $\mu\text{g/ml}$, and (E) 100 $\mu\text{g/ml}$ neutravidin cases. (F) Pixel-by-pixel fluorescence intensity quantification showing the liposome clusters have higher intensities with higher bulk neutravidin concentrations during the fabrication process. Higher fluorescence intensity is related to greater density of tethered liposomes. All scale bars are 20 μm 135

Figure 5.7 Quantification of tethered liposomes on non-patterned and patterned platforms. Particle counting results for single liposomes and liposome clusters on the non-pattern and patterned platforms, respectively. 136

Figure 5.8. Time-resolved tracking of AH peptide interaction with micropatterned viral membrane clusters. (A) Time-lapse fluorescent microscope images show the change in calcein signal when 100 nM AH peptide was added to clusters at $t = 0$ min. During fabrication, the bulk neutravidin concentration was 10 $\mu\text{g/ml}$. (B) Changes in normalized calcein signal intensity of a representative, individual cluster for platforms fabricated using different neutravidin concentrations with the time-resolved release of the calcein marker. (C) Box plot graph reporting trend in calcein release times. Data correspond to experiments presented in (B). Each dot represents one cluster and mean values are reported from Gaussian fitting. (D-F) Corresponding data are presented for the rhodamine signal in the same experimental series. All scale bars are 20 μm . Control indicates non-patterned platform that was fabricated using a 10 $\mu\text{g/ml}$ neutravidin concentration. 138

Figure 6.1. Secondary structure comparison of AH and C5A peptides. Helical wheel projection of (A) AH and (B) C5A amino acid sequences showing an amphipathic distribution of hydrophilic (purple), positively charged (blue), negatively charged (red), and hydrophobic (yellow) amino acid residues. Circular dichroism (CD) spectroscopy

analysis of (C) AH and (D) C5A peptides in the presence of lipid concentrations (with fixed peptide concentration of 100 μM). Insets show the fractional helicity percentages of the two peptides as a function of normalized lipid concentration (defined as lipid concentration over peptide concentration; a value of 20 on the x-axis insets corresponds to 2000 μM lipid, for example). All presented spectra were obtained by averaging three technical replicates, and the helicity percentages were calculated based on mean ellipticity values at the 222 nm wavelengths..... 151

Figure 6.2. Ratio of θ_{222} and θ_{208} values for AH and C5A peptides as a function of normalized lipid concentration. The molar ellipticity ratio of $[\theta]_{222}/[\theta]_{208}$ indicates whether a peptide monomer exhibits predominately linear (single) or coiled-coil helical character. The data are plotted based on the CD spectroscopy results presented in Fig. 6.2. 153

Figure 6.3. Distinct membrane-disruptive potencies of AH and C5A peptides. Time-lapsed fluorescence microscopy images of encapsulated calcein (green, top) and Rh-PE (red, bottom) dye probes for addition of 100 nM peptide to (A) small vesicles (< 125 nm diameter) and (B) large vesicles (> 125 nm diameter), or (C) 10 nM peptide to small vesicles (< 125 nm diameter). Scale bars are 10 μm . (D-E) Corresponding single-vesicle data of peptide-induced vesicle rupture as a function of vesicle size, based on the rhodamine signal. Rupture is defined as when the final fluorescence intensity value of an individual vesicle was 30% or less of the original fluorescence intensity value of that vesicle. Each data point represents an individual vesicle. 155

Figure 6.4. Change in normalized fluorescence intensity of individual, tethered vesicles as a result of membrane-peptide interactions. (A-F) Top: Representative profiles of single-vesicle rupture kinetics for AH and C5A peptides at different peptide concentrations and for different vesicle size ranges. Green and red colors denote calcein and rhodamine signals, respectively. Bottom: Corresponding histogram statistics of membrane permeabilization (green) and membrane lysis (red) time scales, where applicable..... 156

Figure 6.5. Electrochemical impedance spectroscopy analysis of membrane-peptide interactions involving AH and C5A peptides. (A) Schematic illustration of measurement setup. Peptide concentration-dependent changes in G_m (top) and C_m (bottom) signals as a

function of time for (B) AH and (C) C5A peptides, respectively. Peptides were added at approximately $t = 20$ min. 158

Figure 6.6. Structural modeling of AH and C5A peptides and implications for targeting selectivity. (A) Simulated probabilities of helical (red), extended (blue), and random (green) conformations are shown for each amino acid position in the peptide sequences. (B) Highest-ranking conformers of AH and C5A peptides are presented, pointing to distinct conformational properties of the two peptides in bulk solution. (C) Schematic illustration of how AH and C5A peptides interact with lipid membranes. Elements are not drawn to scale. 160

Figure 7.1 Characterization of AH peptide enantiomers. (A) Quartz crystal microbalance with dissipation (QCM-D) measurements of AH peptide-induced rupture of surface-adsorbed vesicles. (B) Circular dichroism spectra of AH peptide enantiomers in deionized water, exhibiting mirror images. 170

Figure 7.2 Single vesicle assay results of AH peptide enantiomers. (A-C) Right: The F.I. of individual vesicles were tracked. Left: Histogram of lag time taken before the rupture of vesicles. (D) Quantification of peptide-induced vesicle rupture as a function of vesicle diameter, where rupture is characterized by the final fluorescence intensity value of individual liposomes after treatment with 100 nM AH-D or AH-L peptide. Each symbol represents data from an individual vesicle. (E) Fraction of ruptured liposomes in different size categories based on the data in (D). An individual liposome is classified as “ruptured” when its final fluorescence intensity is 30% or less of the original fluorescence intensity.¹ 172

Figure 7.3 AH-D peptide exhibits inhibitory effect in primary neuronal cells and in vitro antiviral activity. (A) Left: Effect of AH-D peptide on viability of ZIKV HS-2015-BA-01-infected cells. Right: Production of infectious virus from infected cells. Inhibitory activity of AH-D peptide against viral infection in Vero cells, reported by 50% inhibitory concentration (IC₅₀) values.¹ 176

Figure 7.4 Assessments of therapeutic activity of AH-D peptide in mice.¹ (A) Changes in body mass. (B) Calculated viral load in brain. 178

Figure 7.5 Assessments of therapeutic activity of AH-D peptide in mice.¹ (A) One of cytokine molecules, TNF- α and CCL5, concentrations in brain on day 7 post-infection. (B)

Histopathological scores from hematoxylin and eosin (H&E) staining of brain sections. (C)	
Unidirectional blood-to-brain influx rate.....	179

Abbreviations

AFM	Atomic Force Microscopy
AH	α -Helical
AIDS	Acquired Immunodeficiency Syndrome
ANP	Acyclic Nucleoside Phosphonate
ANTS	8-aminonaphthalene-1,3,6 trisulfonic acid
CD	Cyclodextrin
CD	Circular Dichroism
COVID-19	Coronavirus Disease 2019
DENV	Dengue Virus
dGTP	Deoxyguanosine Triphosphate
DLS	Dynamic Light Scattering
dNTP	Deoxynucleoside Triphosphate
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DPX	<i>p</i> -xylene-bis-pyridinium bromide
EIS	Electrochemical Impedance Spectroscopy
F.I.	Fluorescence Intensity
FRAP	Fluorescence Recovery After Photobleaching
HBV	Hepatitis Virus B
HCMV	Human Cytomegalovirus
HCV	Hepatitis Virus C
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IMP	Inosine-5'-Monophosphate
LEAD	Lipid Envelope Antiviral Disruption
LSPR	Localized Surface Plasmon Resonance
LUV	Large Unilamellar Vesicle
M2	Matrix 2

MERS	Middle East Respiratory Syndrome
M β CD	Methyl- β -Cyclodextrin
NH	Nonhelical
NS	Nonstructural
PERL	Polyunsaturated ER-Targeting Liposome
QCM-D	Quartz Crystal Microbalance with Dissipation
RAFI	Rigid Amphipathic Fusion Inhibitors
Rh-PE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)
RSV	Respiratory Syncytial Virus
RT	Reverse Transcriptase
SALB	Solvent-Assisted Lipid Bilayer
SARS	Severe Acute Respiratory Syndrome
SLB	Supported Lipid Bilayer
SUV	Small Unilamellar Vesicle
tBLM	Tethered Lipid Bilayer Membrane
TIR	Total Internal Reflection
VZV	Varicella-Zoster Virus
ZIKV	Zika Virus

Chapter 1 Introduction

Emerging and reemerging viral pathogens are a major global health problem, and specific therapies and vaccines are unavailable for the vast majority of viral pathogens many medically important viruses possess an Achilles heel, namely a lipid envelope that surrounds virus particles and is a necessary structural component for viral infection. Strikingly, certain antiviral drugs, including specific amphipathic peptides, directly target the viral envelope and cause membrane destabilization, resulting in virus particle lysis and loss of infectivity. This novel antiviral strategy is called Lipid Envelope Antiviral Disruption (LEAD). However, mechanistic details about the membrane destabilization process remain to be understood, and tackling this problem requires innovative experimental strategies. Herein, an antiviral agent-screening platform that integrates soft matter design components together with biomaterial and surface functionalization strategies to facilitate parallel measurements tracking peptide-induced destabilization of nanoscale, virus-mimicking small unilamellar vesicles with tunable size and composition is reported.

1.1 Problem Statement/Hypothesis

Infectious diseases are responsible for 20% of world death totals in 2017 according to Global Burden of Disease study.¹ Infectious or communicable diseases such as respiratory infections, diarrhoeal disease, HIV/AIDS, and malaria are the top leading causes of deaths throughout the past ten years (Figure 1.1). Furthermore, in recent years, multiple viral outbreaks have urged the global community to be prepared against emerging and re-emerging viruses.^{2, 3} Flaviviruses such as dengue estimated to infect 390 million cases per year;⁴ the coronaviruses led to a global outbreak of severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and coronavirus disease 2019 (COVID-19) in 2002, 2012, and 2019, respectively. Although numerous scientists around the world are speeding up the development of vaccines and therapeutics against emerging and re-emerging viruses, going through standard protocols take time without mentioning that haste decision involves safety risks, and virus mutations might make previously effective measurements useless.⁵⁻⁷ Besides from unprecedented pandemics, infectious diseases such as lower respiratory infections, diarrhoeal disease, and HIV/AIDS are among the top leading causes of deaths throughout the past ten years.⁸ Therefore, on the top of the ongoing vaccine/therapy development, a new paradigm of antiviral strategy is needed that exceeds conventional pathogen-specific drug development (*one drug, one bug*) approach.⁹⁻¹¹ Despite numerous efforts, this goal is distant as can be evidenced from the recent SARS-CoV-2 outbreak and there should be more efforts to advance promising classes of universal, broad-spectrum antiviral drugs (*one drug, many bugs*)¹²⁻¹⁵ like penicillin in antibacterial drug classes.

Figure 1.1 Data refers to the specific cause of death as the percentage of total deaths. Note that the total death was estimated to be 56 million in 2017. The red shade in cause of death refers to infectious diseases and the number of deaths was calculated by percentage provided in the graph.

To realize this need, the basic virus structure must be recalled in order to identify potential weak points. By definition, a virus is an obligate intracellular parasite comprising a genetic material (DNA or RNA) surrounded by a protein coat (capsid) and/or lipid bilayers (envelope), which is obtained from the host cell membranes. Virus envelope protects viral genetic material and allows virus infection step by docking and fusing into the host cell.¹⁶ Most of the viral pathogens that cause epidemic infectious diseases are lipid-enveloped viruses, where flaviviruses (dengue, yellow fever, zika, chikungunya, and West Nile), coronaviruses (MERS and SARS), and all the abovementioned viral outbreaks are included.¹⁷⁻¹⁹ The viral envelope derived from the host cell helps the virus to avoid host immune response, which is the main reason why most of the viruses causing epidemic are enveloped viruses.¹⁶ As the most of the protein receptors of virus exist on the lipid envelope, the virus loses its virulence when the integrity of envelope is altered (e.g., change or disruption of the membrane).^{20, 21} Therefore, the viral lipid envelope has drawn attention

as an Achilles heel.²² Recently, Lipid Envelope Antiviral Disruption (LEAD) has been proven to be effective *in vivo*, as evidenced by our therapeutic application of an engineered pore-forming antiviral peptide to successfully treat lethal Zika virus infection in mice.²³ Furthermore, LEAD can be non-clinically tested by lipid model membranes mimicking viral envelopes, which are cost-friendly, safe, and most importantly, providing an opportunity to tune the membrane destabilization activity by engineering peptide sequences. To this end, a successful antiviral drug must specifically target the viral envelope while leaving human cell membranes unperturbed—an important design challenge that motivates this work and selection of appropriate measurement strategies. This thesis highlights the development of LEAD drug screening platform based on biomimetic viral particle, which is broadly useful for evaluating the performance of membrane-active antiviral drug candidates. There are specific amphipathic peptides that can directly target the viral envelope and cause membrane destabilization, resulting in virus particle lysis and loss of infectivity.^{24,25} Taken together, the underscoring hypothesis in this thesis is that this class of antiviral agents can be engineered and applied to multiple families of viruses.

Conventionally, the leakage or degradation of fluorescent-labeled molecules within/from vesicles was widely investigated to characterize the activity of membrane-active peptides such as antimicrobials.²⁶⁻²⁹ However, these approaches are based on ensemble-averaged system (bulk solution) so that the extent of dye leakage induced by vesicle-permeabilizing activity does not always correlate with biological anti-infective activity. This is a crucial limitation as a biological membrane-vesicle model system, which has been reported widely in antibacterial peptides as well as antiviral peptides. Therefore, a novel platform called tethered single vesicle assay has been proposed to mimic the biological condition of virus particles and track the membrane-lytic viral peptides. Since single vesicle tracking can reveal an individual behavior (heterogeneities), which is hidden in averaged signals in conventional methods, utilizing this platform would be beneficial to solve the abovementioned issue. By constructing a robust antiviral peptide screening platform, not only screening therapeutically important peptides but also ligand binding,³⁰ and other confined reactions³¹ can be studied in mechanistic and kinetic details. Another significant feature of single vesicle measurement is the opportunity to track down the size

distribution of vesicles,³² which allows us to explore an influence of vesicle size on membrane partitioning that can be compatibly studied with membrane partition coefficients.^{33,34} To achieve this goal, the following hypotheses underline this thesis:

1. Certain amphipathic, α -helical peptides display lipid vesicle lysing activity, thus studying the structure-function relationship based on membrane-interaction profiles will provide insights to engineer peptides.
2. Tethered single vesicle measurement can reveal mechanistic and kinetic details in membrane-peptide interaction, which are hidden in average signals in ensemble measurements.
3. The lead peptide, AH peptide, selectively lyses lipid vesicles with high membrane curvature, which is an important attribute that can be effectively screened by tethered vesicle assay.
4. The integrated approach using multiple model membrane platforms will provide distinct observations that can link the physicochemical properties and potency of LEAD agents.

1.2 Objectives and Scope

This thesis describes and validates the design, characterization, and application of a tethered lipid vesicle assay that screens potential antiviral agents against enveloped viruses. In order to achieve the objective, this thesis comprises three aims to provide a novel platform to understand the membrane interaction profiles using multiple model membrane platforms and techniques. The following specific aims will provide the motivation for this work:

Aim 1. Improve LEAD agent screening platform design and data analysis by identifying the technical artifacts (dye leakage and imaging-related photobleaching) and addressing each issue with modulating membrane composition and corrective measurements.

Aim 2. Develop virus-mimetic clusters of tethered vesicles that can model the architectural

configuration of clustered viral particles by incorporating micropatterning strategy and compare the observed kinetics obtained from conventional single vesicle platform.

Aim 3. Compare two antiviral peptides by adopting an integrative approach with diverse types of model membranes to investigate the different activities of and further draw structure-function relationships.

1.3 Dissertation Overview

The thesis comprises seven chapters to address the followings:

Chapter 1 Introduction provides the problem statements and hypothesis along with overview of the research scope and describes the rationale that motivates the doctoral thesis topic with key aims and the work scope.

Chapter 2 Literature Review introduces a comprehensive background of conventional antiviral treatment strategy and its challenges followed by the novel concept of lipid envelope antiviral disruption (LEAD) strategy that this thesis underpins. The experimental approaches using model membranes to characterize the profile of membrane-antiviral agents are introduced, which include high-throughput screening, various surface-sensing techniques and tethered lipid vesicle platform.

Chapter 3 Experimental Methodology outlines descriptions of materials, preparation of model membranes, and experimental measurement techniques. The experimental approach also provides detailed introduction of the operating principles.

Chapter 4 Quantitative Accounting of Dye Leakage and Photobleaching addresses the key issues in single lipid vesicle assay, namely dye leakage and photobleaching, along with corrective measures to derive and compare the rate of peptide-induced pore formation. This extended analytical framework is aimed to improve the platform design and data analysis. Various membrane compositions showing different extent of dye leakage emphasizes the

need of suitable normalization procedure to correct the technical artifacts.

Chapter 5 Micropatterned Viral Membrane Clusters reports the development of virus-mimetic clusters of tethered vesicles using micropatterning strategy to print linker molecules selectively onto bioinert surfaces, thereby enabling controlled tethering of biomimetic viral particle clusters across defined geometric patterns. This platform mimics the architectural configuration of clustered viral particles along with significant advantages such as material requirements and fabrication speed.

Chapter 6 Comparing the Membrane Interaction Profiles of Two Antiviral Peptides shows an integrative approach using diverse types of model membranes to investigate the different activities of and further draw structure-function relationships. To address this, multiple surface-sensitive measurement techniques with computational simulations are introduced to explore how the peptide structural properties affect the functional activity and targeting selectivity.

Chapter 7 Discussion and Future Work summarizes the main findings and conclusions that are obtained from the studies and demonstrates critical discussion of underlying scientific concepts that can provide an analytical framework for characterizing antiviral agents. Future directions highlight how these fundamental findings can be further extended to develop improved antiviral strategies utilizing engineering peptide sequence and preclinical studies.

1.4 Findings and Outcomes

The findings obtained in this thesis can lead to a number of outcomes that improve the measurement platform by rationale design and extend the findings to investigate the activity profile of antiviral agents. The novel findings and outcomes of this thesis are as follows:

1. Improving parallel measurements of tethered lipid vesicle platform design and data

- analysis. First, experimental artifacts that might affect the accurate quantitative outcomes were identified as dye leakage and imaging-related photobleaching. The corrective measures were proposed by choosing suitable membrane compositions to minimize the dye leakage while enhancing the biomimetic character of lipid vesicles by adding negatively charged fluorophore and sterols. Second, a data normalization procedure was developed to improve the measurement accuracy. Then, this analytical procedure was applied to determine the rate of peptide-induced pore formation in single lipid vesicles. Up to a three-fold decrease in the measured rate as compared to uncorrected data was identified, thus highlighting the need of corrective measurements.
2. Developing the biomimetic platform that recapitulate the higher-order supramolecular assemblies of viral particles that form cooperative clusters with heightened functionalities. The microcontact printing was utilized to fabricate virus-mimetic clusters of tethered vesicles by printing the linker molecules selectively onto bioinert surfaces. By controlling the linker concentration, the density of tethered vesicles can be tuned while the signal intensity can be increased together. The pore formation and membrane lysis were successfully observed by time-resolved tracking of encapsulated and embedded fluorophores, respectively. This platform is broadly useful for evaluating the potency of LEAD drug candidates within the clusters.
 3. Correlating the structure-function of antiviral peptides by comparing the membrane-interaction profiles. Multiple surface-sensitive measurement techniques along with computational simulations were employed to investigate two antiviral agents, which show distinctive biophysical profiles. The conformational change observed by circular dichroism with series of increasing lipid concentration showed that more extensive helix stabilization in lipid environment might be linked to the greater membrane partitioning, thus greater interfacial activity against lipid membranes. By comparing the results obtained from tethered lipid vesicle and planar bilayer, the selectivity based on membrane curvature was confirmed and this integrative approach manifests the application of model membranes in drug screening.

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Chapter 2* Literature Review

One of emerging broad-spectrum antiviral strategy is to target the lipid envelope of the viruses, which surrounds virus particles in order to protect viral genetic material as well as to aid virus-cell fusion as part of the infection process. This chapter first lay out the background of viruses to introduce conventional, established antiviral strategies along with their mechanisms of actions and corresponding drawbacks. Building on this, the concept of lipid envelope antiviral disruption (LEAD) strategy and previous studies are described, including detailed history of the lead compound of this thesis, AH peptide. Finally, the experimental approaches based on model membranes are outlined to provide a background of the development of tethered lipid vesicle platform.

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2.1 Conventional Antiviral Treatment Strategy

2.1.1 Classification of Viruses

A virus is an obligate intracellular parasite requiring the host cell machinery to replicate. The viral particle comprises a genetic material (DNA or RNA) surrounded by a protein coat (capsid) and/or lipid bilayers (envelope). Therefore, viruses can be classified based on the genetic material as well as the presence of viral lipid envelope. There are seven groups of viruses classified according to viral genome replication (Baltimore classification), which offer direct and useful understanding of viruses as all viruses must go through the synthesis of messenger RNA to produce proteins.¹ Another practical division of viruses is the presence or absence of a host-derived lipid membrane, namely enveloped or nonenveloped viruses, respectively. Viral envelope protects viral genetic material and allows virus infection step by docking and fusing into the host cell.² Most of the circulating viral pathogens are lipid-enveloped viruses, where simplexviruses (causing herpes), flaviviruses (dengue, yellow fever, zika, chikungunya, and West Nile), coronaviruses (MERS and SARS), and lentivirus (HIV) are included.³⁻⁵

As a viral envelope is composed of lipid bilayer, enveloped viruses might be more susceptible to the external environment including humidity⁶ or disinfectants^{6, 7} compared to nonenveloped ones. However, enveloped viruses have other several advantages that can explain their high-profile outbreaks.⁸ First, the enveloped viruses exist from the host cell in conventional secretion pathway (exocytosis) without cell damages and consequent immune responses whereas nonenveloped viruses often cause host cell lysis.⁹ Second, the lipid envelope functions as a cloak to hide the capsid antigens to avoid host immune system.^{10, 11} Lastly, the envelope provides additional compartment to carry more viral proteins, which can also mutate in wider range to escape immune response compared to the nonenveloped viruses' rigid capsid protein.⁸ Therefore, enveloped viruses can adapt to different hosts efficiently by adapting their proteins. As essential the viral membrane is, this may offer a common target for intervention of all enveloped viruses. Table 2.1 outlines clinically important human-infecting enveloped viruses and current vaccine, therapies, as well as the size of viruses.

Table 2.1 Clinically important enveloped viruses and currently available therapy with virus sizes.

Type	Family	Genus	Species	Disease	Vaccination	Target	Size (nm)	Ref.
I DNA (+/-)	Herpesviridae	Simplex virus	Herpes simplex virus 1/2	Oral/Genital herpes	-	DNA polymerase; Entry inhibitor	155–240	12
		Varicellavirus	Varicella-Zoster virus	Chickenpox, shingles	Varivax, Zostavax	DNA polymerase; Entry inhibitor	180–200	13
		Lymphocryptovirus	Epstein-Barr virus	Mononucleosis, lymphoma	-	-	122–180	14
		Cytomegalovirus	Human cytomegalovirus	Mononucleosis, pneumonia	-	DNA polymerase	150–200	15
	Poxviridae	Orthopoxvirus	Variola virus	Smallpox	Dryvax: vaccinia vaccine	-	~300	16
		Parapoxvirus	Orf virus	Contagious pustular dermatitis	Only for non-human animals	-	D: 150–175, L: 220–300	17
IV RNA (+)	Togaviridae	Alphavirus	Sindbis virus (SINV)	Fever, chronic arthralgia	-	-	70	18
			Chikungunya virus (CHIKV)		-	-	66–70	19
			Venezuelan equine encephalitis virus (VEEV)	Venezuelan equine encephalitis (VEE)	Attenuated vaccine	-	~70	20
	Flaviviridae	Flavivirus	Dengue virus (DENV)	Dengue fever	Dengvaxia	-	48	21
			Japanese encephalitis virus (JEV)	Japanese encephalitis	Inactivated JEV	-	50	22
			West Nile virus (WNV)	West Nile fever	-	-	40–45	23
			Yellow fever virus (YFV)	Yellow fever	YF-Vax, Stamaril	-	38	24
			Zika virus (ZIKV)	Fever, rarely microcephaly, Guillain-Barré syndrome	-	-	50	25
		Hepacivirus	Hepatitis virus C (HCV)	Hepatitis C	-	RNA polymerase; Protease; Protein (NS5A); Interferon	50	26
	Coronaviridae	Betacoronavirus	Middle East respiratory syndrome-coronavirus (MERS-CoV)	MERS	-	-	118–136	27
			Severe acute respiratory syndrome-coronavirus (SARS-CoV)	SARS, COVID-19	-	-	60–140	28
	Matonaviridae	Rubivirus	Rubella virus (RuV)	Rubella	Attenuated vaccine	-	50–70	29
	Arenaviridae	Mammaronavirus	Lymphocytic choriomeningitis mammaronavirus (LCMV)	Lymphocytic choriomeningitis (LCM)	-	-	~100	30

			Lassa mammarenavirus (LASV)	Lassa hemorrhagic fever	-	-	~120	31
V RNA (-)	Orthomyxoviridae	Alphainfluenzavirus	Influenza A virus	Influenza	New versions of the vaccines are developed twice a year [‡]	RNA polymerase; Endonuclease; Protein (M2); Neuraminidase	80–120	32, 33
	Paramyxoviridae	Morbillivirus	Measles virus (MeV)	Measles	Measles, mumps, and rubella (MMR) vaccine	-	220	34
		Orthorubulavirus	Mumps orthorubulaviruses (MuV)	Mumps		-	100–600	35
		Henipavirus	Nipah virus (NiV)	Nipah virus infection	-	-	~500	36
	Pneumoviridae	Orthopneumovirus	Human respiratory syncytial virus (HRSV)	Respiratory tract infections	Prophylaxis: Palivizumab	RNA polymerase; Entry inhibitor	150	13
	Phenuiviridae	Phlebovirus	Rift Valley fever phlebovirus	Rift Valley fever (RVF)	-	-	44.7	37
	Rhabdoviridae	Lyssavirus	Rabies lyssavirus	Rabies	Killed/Inactivated vaccine	Antibody (Prophylactic, therapy): Rabies immunoglobulin	D: 75, L: 180	38
		Tibrovirus	Bas-congo virus (BASV)	Hemorrhagic fever	-	-	D: 60–85, L: Vary	39
	Peribunyaviridae	Orthobunyavirus	Bunyamwera orthobunyaviruses (BUNV)	Bunyamwera fever	-	-	90–125	40
	Nairoviridae	Orthonairovirus	Crimean-Congo hemorrhagic fever orthonairovirus (CCHFV)	Crimean-Congo hemorrhagic fever (CCHF)	-	-	80–120	41
VI RNA (+)	Retroviridae	Lentivirus	Human immunodeficiency virus 1 (HIV-1)	Acquired immune deficiency syndrome (AIDS)	Prophylaxis: Emtricitabine/tenofovir	DNA polymerase; Reverse transcriptase; Protease; Integrase; Entry inhibitor	125	43
		Ebolavirus	Ebola virus (EBOV)	Ebola virus disease (EVD)	Attenuated virus: VSV-EBOV vaccine	-	D: 80, L: Vary	42
VII DNA (+/-)	Hepadnaviridae	Marburg virus	Marburg virus (MARV)	Marburg virus disease	-	-	Dr: 80, L: Vary	42
		Orthohepadnavirus	Hepatitis B virus (HBV)	Hepatitis B	Recombivax HB, Engerix-B	DNA polymerase; Reverse transcriptase; Interferon	45	44

[‡]The intradermal vaccine was not available during the 2018–2020 influenza seasons.

2.1.2 Classification of Established Antiviral Therapies

About 12% of existing antiviral drugs can treat more than one viral diseases because many medically important viruses share similarities in their replication cycles.⁴⁵ In other words, antiviral drugs from the same drug group exploit the common mechanisms of action to impede viral replication. Therefore, repurposing of approved drugs is an effective

strategy when novel viruses emerges. For instance, remdesivir/favipiravir (viral RNA polymerase inhibitor), ivermectin (anti-parasitic agent that inhibit viral protease), and hydroxychloroquine (antimalarial agent that block viral-cell fusion) were repurposed to undergo clinical tests against SARS-CoV-2 since their safety has been already approved.⁴⁶ The classification of current antiviral therapies in this thesis is listed based on the inhibitory target sites such as viral replication, entry/release, and others including immune system modulators (Figure 2.1).

Figure 2.1 Classification of established antiviral strategies. Replication inhibitors stop viral genome replication within infected host cells whereas entry inhibitors target existing virus particles before entry to the host cells or right after the release. The figure is adapted from Ref ⁴⁷.

2.1.2.1 Replication Inhibitor

Replication inhibitors include viral enzyme inhibitors and other viral protein inhibitors. For example, in one of the most widely studied HIV case, the targeted viral enzymes are DNA/RNA polymerase, reverse transcriptase (RT), protease, and integrase. The other viral proteins may include nonstructural proteins such as NS5A/5B in HCV, matrix 2 (M2) protein in influenza. As can be seen from the approved drugs (mainly for the treatment of HCV/HBV and HIV), the inhibition of viral enzymes has been demonstrated as the most effective.

The DNA polymerases are critical component in the replication of DNA as well as retroviruses comprising type I, VI, and VII, thus several antiviral drugs were developed to target this machinery. Most of the approved antiviral drugs are nucleoside/nucleotide analogues inhibiting viral genome replication. As for enveloped viruses, *Herpesviridae* families (type I), HIV (type VI), and HBV (type VII) utilize this strategy. First, nucleoside analogues turn into triphosphate (active) form to target the viral DNA polymerase by competing with natural deoxynucleoside triphosphates (dNTPs) substrates and/or incorporating into the growing DNA chain. Acyclic guanosine analogues including acyclovir, ganciclovir, penciclovir, famciclovir, valacyclovir, and valganciclovir are mostly used to treat *Herpesviridae* such as herpes simplex virus (HSV), varicella zoster virus (VZV), and human cytomegalovirus (HCMV). The last three drugs, famciclovir, valacyclovir, and valganciclovir, are prodrugs derived from penciclovir, acyclovir, and ganciclovir, respectively, to increase bioavailability. All drugs share similar mechanism of action, which is to compete with the deoxyguanosine triphosphate (dGTP) of viral DNA polymerase to inhibit viral DNA synthesis. After the uptake of acyclic nucleoside analogues, they need to go through three steps of phosphorylation (triphosphate form) to interact as competitive inhibitors or act as DNA chain terminator. In order to complete this stage, specific viral kinases are needed, whereby many other DNA viruses do not have except for *Herpesviridae*. Although acyclovir has been considered as gold standard, more recently developed famciclovir is also widely used to treat HSV and VZV infections^{48,49} as acyclovir administration schedule is short and acyclovir-resistant strains of herpes have emerged.⁵⁰

Acyclic nucleoside phosphonate (ANP) analogues are more recently developed drug

class that share similarities with acyclic guanosine analogues as well as differences. First, the ANPs already contain a phosphate-mimetic group. In other words, ANPs require only two phosphorylation steps to achieve the active metabolite stage, thus they are not dependent on the viral kinase, which is needed for acyclic guanosine analogues.⁵¹ In addition, they have a linkage of phosphonate (P-C-O) instead of phosphate (P-O-C). The phosphonate linkage, unlike phosphate, is not affected by the hydrolase enzyme esterase, thus the molecule will in the DNA chain and provide irreversible DNA chain termination.^{51, 52} Therefore, overall, ANPs show higher bioavailability and stability with broad-spectrum among DNA viruses as well as HIV infections.

Another interesting drug class that might be classified here is ribavirin, which is a nucleoside analogue that is effective against multiple RNA viruses including HCV (type IV), influenza, and respiratory syncytial virus (RSV, type V) as well as some DNA viruses. The most popular usage of ribavirin is to combine with interferons for HCV treatment rather than monotherapy. While ribavirin triphosphate can interact with multiple types of viral RNA polymerases, the mechanism(s) of action is not clear.⁵³ Therefore, it is conceived that the mechanisms of ribavirin's inhibitory effect might be different from viruses rather than sharing one universal mechanism.^{53, 54} For example, the inhibition of inosine-5'-monophosphate (IMP) dehydrogenase, alterations of intracellular nucleotide concentrations,⁵⁵ and immunosuppressive effects have been reported and all the factors may have contributed to HCV combination therapy.⁵⁶ Although there is controversial reports on the ribavirin triphosphate's role in the chain termination,⁵⁷ it has been shown to selectively inhibit the RNA polymerase of influenza virus.⁵⁸ Similarly, favipiravir triphosphate has been shown broad-spectrum inhibitory activities against RNA polymerases of influenza and other RNA viruses and clinical trials are going on with SARS-CoV-2.⁵⁹

Protease (HCV and HIV), integrase (HIV), and more recently, endonuclease (influenza) are other viral enzymes that catalyze the processing of viral replication and maturation, thus they are attractive targets followed by DNA/RNA polymerases. Inhibitors of enzymes initially brought dramatic efficacy and improved the clinical treatments. However, the rapid emergence of protease inhibitors-resistant viral strains was observed for the treatment of HIV-1 and HCV infection.⁶⁰ For example, some of HCV protease inhibitors designed based on the HCV genotype 1 are active only against genotype 1. Additionally,

there are inhibitors blocking other viral proteins such as HCV nonstructural (NS) proteins or influenza matrix proteins. There are four approved HCV NS5A inhibitors (daclatasvir, ledipasvir, ombitasvir, and elbasvir) and two influenza matrix 2 (M2) inhibitors (amantadine, and rimantadine). Although the class of antiviral replication inhibitors are broad, diverse, and clinically useful,⁴⁵ the viral genome replication is high error-rate process, thus the generation of variants with antiviral resistance is found frequently.

2.1.2.2 Entry/Release Inhibitor

Entry inhibitors are different from replication inhibitors in the sense of mechanisms as well as general structures. There are seven FDA-approved entry inhibiting drugs, including one HSV drug (docosanol), two RSV antibody drugs (palivizumab and human immunoglobulin), two VZV antibody drugs, and two HIV drugs (enfuvirtide and maraviroc). As for release inhibitors, 4 neuraminidase inhibitors including Tamiflu (oseltamivir) work against both influenza A and B are the representative case. Neuraminidase is one of membrane glycoprotein, playing a critical role in viral propagation from infected cells by cleaving the sialic acid. The design of inhibitors were inspired by sialic acid to block the active site of neuraminidase.⁶¹ While neuraminidase inhibitor-resistant variants appeared in quite low rate in adults (0.4%) rather than children (4–8%), much higher rates (~20%) have been also reported.⁶²

As for the entry inhibitor, the most common types are antibody drugs because they recognize a unique moiety of viral proteins (antigen) and prevent the viral attachment. Antibodies or immunoglobulins are proteins, which, originally, produced by plasma cells to neutralize pathogens and sustain the immune system.⁶³ Another type of entry inhibitors enfuvirtide and maraviroc, acting against HIV. Enfuvirtide is a 36 amino acids-length biomimetic peptide, which was designed based on one of components of the HIV-1 fusion machinery. HIV-1 binds to the host cell using the viral transmembrane protein gp120; gp41, which trigger a conformational change to assist the fusion. Maraviroc, on the other hand, acts against host cell's chemokine receptor (CCR5: protein on the surface of white blood cells), thereby inhibiting the gp120 protein's attachment to the host cell. However, both

entry inhibitors are limited to certain cases; enfuvirtide is only effective against HIV-1⁶⁴ whereas maraviroc should consider HIV tropism.^{65, 66}

Lastly, 10% docosanol (saturated 22-carbon alcohol) is another interesting drug class that is considered broad-spectrum against enveloped viruses to treat recurrent fever blisters or cold sores caused by mostly HSV. As the structure implies, docosanol is highly lipophilic and the topical administration allows excellent tissue penetration and is regarded safe. The mechanism of action is yet equivocal but the evidence of interference against viral fusion, transmembrane migration (cytoplasm entry), and migration to the nucleus of host cells were reported, thus one or more interference in the common pathways of viral entry is suspected.⁶⁷ Also, the incubation of HSV with docosanol did not show effect in its infectivity, demonstrating the lack of any direct antiviral effect. Notably, acyclovir-resistant HSVs can be treated with docosanol.⁶⁸

2.1.2.3 Challenges of Antiviral Drug Development

As of 2016, 90 antiviral drugs were approved to treat nine infectious diseases, whereby >85% of the therapy target HIV, HCV, herpesvirus, and influenza virus.⁶⁹ Nevertheless, a definitive cure for these viruses is yet to be discovered (*e.g.*, limited to certain viral strains in HIV⁷⁰ and the most of HCV inhibitors are only allowed against genotype 1⁶⁹) and more than 200 human viral diseases are remained to be absence of any antiviral drug. The antiviral drug development start from enzyme (target-specific), structure-based design, or re-purposing drugs, whereby oftentimes expand based on the lead compounds that already have been proven effective.⁷¹ In consequence, about five newly developed drugs since 2016 target the same group of viruses as mentioned above. For many infectious diseases, the drug development is encumbered by not only a lack of initiative compound but also insufficient platform to understand and test within cellular or animal models.⁷¹

Considering unpredictable outbreaks of emerging and re-emerging infectious disease, broad-spectrum antiviral drugs are gaining attention. By principle, broad-spectrum antivirals act against at least two different families of viruses by a common mechanism of action, thus adequate for responding to emerging infectious diseases. Abovementioned

ribavirin (classified as inhibitor of RNA polymerase but mechanisms are equivocal) is one of broad-spectrum antivirals approved early in 1971 and is commonly used in combination therapy. However, side effects caused by nucleoside inhibitors are recognized to be serious by causing lipid abnormalities,⁷² and central nervous system disturbances.⁷³ Taken together, the emergence of drug-resistant viruses is the main issue to be tackled.

2.2 Lipid Envelope Antiviral Disruption (LEAD) Strategy

One of emerging broad-spectrum antiviral strategy is to target the lipid envelope of the viruses, which surrounds virus particles in order to protect viral genetic material as well as aid virus-cell fusion as part of the infection process.⁷⁴ For these reasons, disrupting structural properties of the lipid envelope, such as inducing membrane lysis or causing a decrease in membrane fluidity, can compromise viral infectivity (Figure 2.2).^{75, 76} In other words, the drugs targeting the integrity of lipid membrane of the envelope are included whereas the drugs targeting proteins that exist on lipid envelope (e.g., viral glycoproteins involving in the cell entry) are classified as conventional antiviral drugs. An important advantage of LEAD strategy is the emergence of drug-resistant viruses is almost not possible because the viral lipid envelope is derived from host cell membranes and not encoded within the viral genome.⁷⁷ Recent findings have further demonstrated that Lipid Envelope Antiviral Disruption (LEAD) is effective *in vivo*, as evidenced by the recent therapeutic application of an engineered amphipathic α -helical (AH) peptide to successfully treat lethal Zika virus infection in mice.⁷⁸ The key premise of this thesis is that the LEAD strategy can be non-clinically tested by lipid model membranes mimicking viral envelopes, which are cost-friendly, safe, and most importantly, providing an opportunity to tune the membrane destabilization activity by engineering structures of drugs (e.g., peptide sequences).

Figure 2.2 The LEAD strategy is to target and disrupt the viral membranes, which can be modeled by artificial lipid membrane and effective against various families of enveloped viruses.

2.2.1 Classes of LEAD Antiviral Drugs

The first drug class is lipophilic photosensitizers, initially reported as LJ001, which can intercalate into lipid membranes, absorb visible light wavelengths to produce singlet oxygen free radicals ($^1\text{O}_2$), and damage membrane phospholipids of viruses.^{79, 80} The $^1\text{O}_2$ oxidizes unsaturated phospholipids into hydroxylated fatty acid species, stabilizing positive spontaneous curvature with decreased membrane fluidity, then finally, inhibiting virus-cell membrane fusion and viral entry.^{14, 79, 80} As viruses cannot repair the lipid membrane, once damaged membranes were irreversible but the toxicity could be aroused from the fatty acid synthesis intervention and modulation of membrane fluidity. Moreover, the light-dependent activation has limited its application to non-mammalian hosts such as aquatic species and further studies have shown antiviral effect of LJ001 *in vitro* and *in vivo* when the viruses are preincubated or horizontally transmitted with the agent. More recently developed homologs, JL122 and JL118, have shown greater *in vitro* potency/efficacy, better tissue penetration and bioavailability.⁸¹ All series of this class were further studied to be applied in aquaculture industry for water-immersion antiviral treatments due to light-dependency.

Similar to LJ or JL series compounds, there are rigid amphipathic fusion inhibitors (RAFIs), which can alter the membrane curvature⁸² but not affecting membrane fluidity.⁸³ The inverted-cone-shaped RAFIs consist of planar and rigid hydrophobic moiety and relatively large hydrophilic head group (Figure 2.3A). They are also a type of

photosensitizer generating radicals in solution such as LJ/JL series. The advanced feature of RAFIs compared to LJ/JL series is that the intercalation within viral envelope alone can inhibit the formation of negative curvature of viral membrane and block virion-cell fusion. The leading template of RAFI is called aUY11 with uracil-arabinose polar moiety connected with ethynyl-perylene hydrophobic moiety. Based on the structure of aUY11, modified derivatives (*e.g.*, dUY11, ddUY11, 1–12a and b, respectively, and 13UY11) were developed and characterized to compare their antiviral activity as well as to investigate the mechanism of action.^{77, 84} As a result of these studies, the mode of action of RAFIs are not dependent on specific chemical groups but more dependent on the inverted-cone-shape itself.⁴² Although the majority of RAFI derivatives showed acceptable cytotoxicity ($CC_{50} > 20 \mu M$),⁴² further studies would be needed to rationally design therapeutics based on the lead molecule and optimize their applications.

Another interesting drug class involves targeting sterols such as cholesterol, which is important component to regulate the rigidity and fluidity of lipid membrane. The virus-cell fusion occurs when viruses dock onto the host cell membrane using viral receptors and trigger conformation changes of both membranes. Therefore, the alteration of lipid composition at the interface of virus-cell membranes can affect the fusion process. In addition, the extraction of cholesterol can simply lead to disorganization of lipid raft and subsequent dissociation of membranous proteins. Likewise, as many viral entry and exit routes involve cholesterol-enriched regions or lipid rafts of the host cell plasma membranes, changes in cholesterol concentrations can also alter host cell susceptibilities to viral infections.^{85, 86} The level of cholesterol might assist or block the infection depending on the viruses; the high level of cholesterol might enhance the infectivity of alphavirus such as Sindbis virus⁸⁷ whereas the addition of cholesterol readily blocks flavivirus-induced infection.⁸⁸ Although these examples sound equivocal, the gist is that the disruption of cholesterol homeostasis either by removal or addition of cholesterol will affect the infectivity. The use of cholesterol-depleting agents such as methyl- β -cyclodextrin (M β CD)^{89, 90} and polyunsaturated ER-targeting liposomes (PERLs)⁹¹ showed reduced infectivity, likely due to the disturbance in cholesterol-enriched lipid-ordered membrane domains (or lipid rafts) of cellular and/or viral membrane.

Figure 2.3 Molecular structure of RAFI with schematics of mechanism of action (A), cyclodextrin compounds (B), and CLR01 (C). The cartoon from panel (A) is adapted from Ref ⁹².

More recent study showed that the cyclodextrins (CDs) modified with heparan sulfates mimickers can irreversibly damage the viruses (HSV, RSV, ZIKV and HIV) with high barriers to resistance.⁹³ The inhibitory effect was the strongest during the preincubation and moderately active during the parallel administration of viruses with modified CD (Figure 2.3B). Interestingly, a cholesterol replenishment assay showed that the additional presence of cholesterol negligibly affects the inhibitory activity of the compound, thus suggesting that the cholesterol extraction from membrane is not a main mechanism of action. Also, a DNA exposure assay showed that the incubation with modified CD and HSV induced genome leakage, which indicates the disruption of the virus structure, likely including viral envelope. It has been also found that the heparin sulfates function as initial attachment receptors and affect the viral protein. As for molecular structure, the sufficient linker length between CD and the sulfonate group has been found to be one of the important parameters.⁹⁴ Taken together, the mechanism(s) of modified CDs is not yet clear but fundamental study using model membranes starting from simple

composition will be exciting to elucidate mechanism as well as to optimize the modification. Another promising application of CDs include mouthrinses combined with other active ingredients.⁹⁵ Nevertheless, the attempt to alter viral membrane may influence the changes in the plasma and cellular membrane cholesterol, which might disturb the normal signal transduction activity by altering cholesterol-enriched lipid rafts and caveolae⁹⁶ and modulating immune responses.⁹⁷

Similar to cholesterol-involved strategy, CLR01, molecular tweezer (Figure 2.3C), can selectively interact with lipid-raft-rich domains, causing viral membrane disruption in enveloped viruses including HIV-1, HCV, Zika virus, Ebola virus and other *Herpesviridae* viruses including HSV-1 and HCMV.^{98, 99} In addition, CLR01 is active in the presence of biological fluids including saliva, semen, and urine but not in the presence of serum, which might be suitable as a topical microbicide. CLR01 was originally developed as a tweezer to bind positively charged amino acids such as lysine and arginine residues. This characteristic has been exploited to disrupt noncovalent molecular interactions in abnormal self-assembly (*e.g.*, amyloid aggregates).¹⁰⁰ However, for the mechanism of antiviral activity was, surprisingly, due to interaction between CLR01 and lipid-raft-rich domain in the viral envelope. The tweezer preferentially bound to the sphingomyelin/cholesterol-rich membrane, which was verified by model membrane permeability experiments.⁹⁹ By combining the virucidal activity of CLR01 in semen with anti-amyloid effects and particularly elevated amount of sphingomyelin/cholesterol in HIV membrane, this molecular tweezer can be a promising topical microbicide against HIV.

2.2.2 Amphipathic α -Helical (AH) Peptides

Membrane-active peptides are important anti-infective agents that target infectious pathogens by destabilizing phospholipid membrane coatings.¹⁰¹⁻¹⁰³ The classical description of anti-infective peptides typically focuses on cationic antimicrobial peptides that selectively destabilize membranes enriched in negatively charged lipids (*e.g.*, bacterial cell membranes).^{104, 105} In recent years, there has been increased attention on another exciting class of anti-infective peptides termed antiviral peptides, which can directly impair the lipid membrane surrounding enveloped virus particles.¹⁰⁶ Unlike the cationic

antimicrobial peptides described above, two of the most widely studied, membrane-active antiviral peptides, the 27-mer AH and 18-mer C5A,¹⁰⁷ have net charges of 0 and -2 , respectively. Interestingly, the AH and C5A peptides have partially overlapping amino acid sequences and are both derived from the N-terminus of the hepatitis C virus (HCV) nonstructural protein 5A (NS5A) which plays an important role in HCV genome replication, as illustrated in Figure 2.4.

Figure 2.4 Biological origin of the antiviral AH and C5A peptides, which correspond to the N-terminal amphipathic helix of the HCV NS5A protein.

Originally, the AH peptide was designed as a synthetic analogue to mimic the NS5A N-terminal amphipathic helix of an HCV genotype 1b strain (AH is an abbreviation for amphipathic, α -helix) for at least two reasons: (i) NS5A membrane association is necessary for HCV genome replication and dependent on the NS5A N-terminus¹⁰⁸; and (ii) it is difficult to express and purify full-length NS5A protein due to the amphipathic character of its N-terminus (it is often expressed without the N-terminus; see, *e.g.*, Refs. ¹⁰⁹⁻¹¹¹). For these reasons, the AH peptide provided a useful model system to investigate the molecular determinants potentially influencing NS5A membrane association to model phospholipid and cell-derived membrane platforms.¹¹²⁻¹¹⁴

In the course of these studies, it was serendipitously discovered that the AH peptide can rupture lipid vesicles below a certain diameter (~ 160 nm; see Refs. ^{115, 116}) and follow-up studies indicated that this membrane-disruptive behavior relates to membrane curvature-dependent pore formation and resulting strain-induced membrane lysis.¹¹⁵⁻¹¹⁸ Interestingly

and in contrast to cationic antimicrobial peptides, the AH peptide has also been shown to disrupt lipid membranes largely independent of membrane surface charge.¹¹⁹ Also, previous study has proven that the amphipathic nature and/or α -helical character of AH peptide is crucial by testing a control, nonhelical (NH) peptide, in which three point amino acid mutations disrupt its hydrophobic face (Figure 2.5).

Figure 2.5 Helix net diagrams of AH, NH, and C5A peptides. The hydrophobic face is indicated with long continuous stretch of yellow shade in AH and C5A peptides whereas no sustained hydrophobic face was observed in NH peptide. The substituted or mutated amino acids compared to AH peptide are indicated as blue letters.

While it remains unclear how the membrane-disruptive activity of the isolated AH peptide might relate to supporting HCV genome replication, it has been demonstrated that the AH peptide's membrane-disruptive activity works *in vitro* against numerous, medically important enveloped viruses such as Zika and Dengue along with HCV itself. Using various nanoscale characterization techniques, it has been shown that AH peptide treatment can disrupt virus particles.^{115, 120} Importantly, due to its membrane curvature-dependent mechanism, AH peptide exhibits high potency against enveloped viruses (half-maximal efficacy in the range of 10 nM – 1 μ M depending on the virus and assay) while exhibiting low cytotoxicity against larger mammalian cells (half-maximal cytotoxicity around 50 μ M or higher depending on the cell type and assay).¹²¹ An engineered version of AH peptide that is composed of all D-amino acids was recently shown to work *in vivo* to therapeutically treat lethal Zika virus infection in mice by reducing viral loads and blunting virus-related inflammation, and the peptide was also able to cross the blood-brain barrier.¹²¹ While it is primarily thought that the AH peptide works against extracellular virus particles, it has been speculated that it might also inhibit intracellular NS5A membrane association through competitive binding to cellular receptors.¹⁰⁸

A few years after the AH peptide was first designed, the C5A peptide was discovered through a high-throughput screen that investigated the potential anti-HCV activity of 441 synthetic peptides that were derived from the genome of an HCV genotype 1a strain.¹⁰⁷ Early work showed that the C5A peptide has potent antiviral activity against numerous enveloped viruses, including HCV, HIV, Dengue, and Herpes simplex (half-maximal efficacy in the range of 500 nM – 5 μ M depending on the virus and assay).^{107, 122-124} On the other hand, it was also reported that C5A peptide exhibits greater hemolytic activity than AH peptide,¹²⁵ and thus its ongoing development has focused on topical microbicide applications. To date, C5A peptide has been shown to protect mice¹²⁶ and non-human primates¹²⁷ against HIV transmission *ex vivo*. The current understanding is that C5A peptide also disrupts enveloped virus particles although it has also been shown to exhibit immune-stimulating activities, suggesting that it might be a useful adjuvant as well.¹²⁸ From a mechanistic perspective, an important distinction between the AH and C5A peptides is that the C5A peptide does not exhibit membrane curvature selectivity and C5A peptide can rupture lipid vesicles independently of vesicle size, which helps to explain why it has less

targeting selectivity.^{122, 125} At the same time, such functional differences motivate deeper investigation of the membrane-interaction profiles of these two antiviral peptides and how functional differences might be related to variations in peptide conformational structure and membrane-induced peptide conformational changes. Indeed, comparison of the aligned AH and C5A sequences reveals that the two peptides are structurally similar at the primary amino acid level and most amino acid differences between the two aligned sequences are conservative substitutions, which is consistent with the fact that the NS5A AH is conserved across HCV genotypes, including the 1a and 1b genotypes that have ~80% genetic similarity overall.^{129, 130} This similarity points to the likely importance of peptide conformational properties in driving membrane interactions and addressing this topic forms the focus of the present study (Table 2.2).

Table 2.2 Comparison between AH and C5A peptide.

	AH peptide	C5A peptide
Origin	HCV NS5A 1b	HCV NS5A 1a
Sequence	SGSWLRDVWDWICT VLTDFTWLQSKL	SWLRDIWDWICE VLSDFK
Molecular weight	3283.75	2310.63
Isoelectric point	8.58	4.32
Net charge	0	-2
Hydrophobicity	0.69	0.74
Hydrophobic moment	0.47	0.68
Discovery of antiviral activity	Membrane-disruptive behavior observed from lipid vesicles below ~160 nm	High-throughput screen investigating the potential anti-HCV activity
<i>in vitro</i> test	HCV, ZIKV, DENV, CHIKV, YFV, JEV	HCV, HIV, DENV, Herpes simplex
<i>in vivo</i> or <i>ex vivo</i> test	Protection against lethal ZIKV infection in mice; Crossed the blood-brain barrier	Topical microbicide to protect non-human primates from HIV transmission
Efficacy range	10 nM – 1 μ M	500 nM – 5 μ M
Minimal hemolytic concentration (MHC)	50 μ M	12.5 μ M

2.3 Experimental Approaches Using Model Membranes to Evaluate Antiviral Agents

Biological membranes consist of lipid bilayer, separate and protect cells from the external environment by allowing selective permeabilization. They also host the membrane proteins that can communicate and transport chemicals/ions. Since the structure of biomembranes are complicated, simplified artificial model membranes, mimicking vital characteristics of biomembranes, enable controlled and reproducible alternative platform to study the biophysical dynamics in the interface. Simplified model membranes can be precisely defined to incorporate necessary parameters and systematically evaluated while recapitulating the dynamics of biological membranes. To facilitate the characterization, model membrane systems are often studied with several platforms including bulk suspension, surface-sensitive measurements and tethered lipid vesicles.

2.3.1 Model Membranes to Mimic Biological Membranes

There are two main types of model membranes: (1) planar lipid bilayer and (2) lipid vesicle platform (Figure 2.6). The structure of planar bilayer is flat and uniform, exhibiting two-dimensional lateral mobility.¹³¹ The most common form of planar bilayer system is supported lipid bilayer (SLBs) that self-assemble on solid substrates by conventional vesicle fusion or solvent-assisted lipid bilayer (SALB) method.¹³² The SLBs are physically stabilized by intimate association with the supporting substrate and lower leaflet, thus offering variety of opportunities to use surface-sensitive measurement techniques.^{133, 134} However, this rigid platform lacks an ionic reservoir as the membrane is closely associated with the surface, thus limiting the application involving incorporated protein, permeability of membrane-active agents, and membrane transport functions.^{135, 136} Tethered lipid bilayer membranes (tBLMs) compensate inherent drawbacks of SLBs as aqueous reservoirs exist on each side of the bilayer and can host transmembrane proteins as well as maintain two-dimensional fluidity.¹³⁷ Also, tBLMs can be monitored by electrochemical impedance spectroscopy (EIS) to characterize the membrane sealing properties with resistance and capacitance of the ionic reservoir.

Lipid vesicles are spherical bilayer structures with an aqueous internal space.

Depending on the balance between the vesicle-substrate interaction and osmotic pressure, vesicles can either undergo rupture-fusion to form SLBs or adsorb onto the surface without rupture. It should be noted that the vesicles are soft matter, which can be deformed when they adsorb onto the surface and the extent of shape deformation often depends on the experimental conditions such as salt concentration, lipid compositions, vesicle size or solid support.¹³⁸⁻¹⁴⁰ Therefore, the adsorbed layer of vesicles are pancake-like with irregular morphologies rather than spherical shapes. As the interior as well as the layer of lipid vesicles are hydrodynamically coupled with aqueous solvent, the intact vesicle layer exhibits viscoelastic properties.

AH peptide was extensively studied using abovementioned platforms with various measurement techniques that will be discussed in the next section. For instance, the potential of antiviral activity of AH peptide was first discovered while studying binding dynamics of AH peptide using artificial membranes.¹⁴¹ When AH peptide was added to intact, adsorbed vesicle layer, it induced spontaneous rupture of these vesicles and transformed the lipid assemblies into SLB. In another study, it was found that AH peptide significantly binds to cell-derived membranes whereas negligibly binds to artificial phospholipid membranes, thus suggesting AH peptide interacts with receptor protein rather than planar lipid membrane itself.¹⁴² Followed by this observation, model membranes were used to find out curvature-sensing ability of AH peptide and its analogues^{78, 143, 144} and to engineer the peptide therapeutics. Previous literatures that studied AH peptide and abovementioned platforms with various measurement techniques are presented in Table 2.3. Likewise, it is important to determine what types of model membranes can assist to elucidate interaction kinetics and design the platform.

Figure 2.6 Various simplified model membrane platforms to mimic complex biological membrane.

Table 2.3 Previous literatures that studied AH peptide and various model platforms with various measurement techniques that will be discussed in the next section.

Platform	Measurement Technique	Findings	Reference
SLB, SUV	AFM, fluorescence microscopy (FRAP)	Amphiphilic AH peptide was employed to immobilize lipid molecules, thus creating lipid partition in pattern.	Langmuir, 2007 ¹⁴⁵
SLB	QCM-D	AH peptide significantly binds to cell-derived membranes whereas negligibly binds to artificial phospholipid membranes, thus suggesting AH peptide interacts with receptor protein.	J. Virol., 2007 ¹⁴²
SUV	QCM-D, AFM	Vesicle-to-bilayer transformation by AH Peptide on gold and titanium oxide surface.	J. Am. Chem. Soc., 2007 ¹⁴¹
SUV	QCM-D, reflectometry	First combination of acoustic-based sensor and optical detection technique to follow the transformation of lipid assemblies by tracking hydration signature.	Anal. Chem., 2009 ¹⁴⁶
SUV/LUV*	QCM-D, AFM	AH peptide's vesicle size dependency was found using QCM-D. AFM was utilized to probe that AH peptide treatment to HCV particles cause rupture.	ACS Chem. Biol., 2009 ¹⁴⁷
SUV/LUV	Single vesicle assay	The mechanism of AH peptide-induced pore formation and membrane lysis depending on membrane-curvature was first studied using single vesicle assay.	Nano Lett., 2012 ¹⁴³
SUV/LUV	QCM-D	AH peptide binds cooperatively to small vesicles only, suggesting that the binding interaction occurs via a different mechanism depending on the vesicle sizes.	J. Phys. Chem. B, 2013 ¹⁴⁸
SUV	QCM-D, ellipsometry	AH peptide promote the rupture of SUVs to form positively and negatively charged bilayers on TiO ₂ , whereby the transformation of negatively charged ones were relatively quicker than positively charged ones.	J. Phys. Chem. B, 2014 ¹⁴⁹
LUV	QCM-D	AH peptide binds specifically to PI(4,5)P ₂ , inducing a conformational change. Note the vesicle used here was 400 nm-extruded.	Gastroenterology, 2015 ¹¹⁴
SUV/LUV	Tryptophan florescence, single vesicle assay	AH and C5A peptides were compared to study correlations between vesicle size dependent membrane-partitioning and corresponding functional activity using single vesicle assay.	Small, 2015 ¹²⁵
GUV, LUV	Fluorescence microscopy (with FRAP)	AH peptide induces microdomain formation in cholesterol-containing membranes whereas it undergoes global softening (at high concentration) in cholesterol-depleted membranes.	Biophys. J., 2015 ¹⁵⁰
SUV	QCM-D, ellipsometry	By combining two surface-sensing techniques, the critical surface bound peptide-to-lipid ratio inducing membrane lysis was calculated, which is nearly constant and much higher than the one required for the onset of pore formation.	J. Am. Chem. Soc., 2016 ¹⁵¹
SUV	QCM-D, SPR	Nanohole array to trap single SUV was developed, and then AH peptide-induced rupture was observed without SLB formation on the sidewalls.	Small, 2016 ¹²⁰

SUV	QCM-D, LSPR	Utilized the sensing depth differences between LSPR and QCM-D. During AH peptide-induced SLB formation, LSPR detected an increase in optical mass (SLB) near the sensor surface whereas QCM-D detected a significant loss in net acoustic mass (coupled solvent).	Anal. Chem., 2016 ¹⁵²
SUV	Voltammetry	First use of voltammetry to detect SUV rupture induced by AH peptides.	Anal. Chem., 2017 ¹⁵³
SUV/LUV	QCM-D, single vesicle assay	The curvature sensing of AH-L and -D peptides (melittin as a control) were studied for biophysical design before therapeutic evaluation and <i>in vivo</i> studies.	Nat. Mater., 2018 ⁷⁸
SUV/LUV, tBLM	Single vesicle assay, EIS	Combined with circular dichroism spectroscopy and computational simulation, peptide's structure–function relationship was studied.	Langmuir, 2019 ¹⁴⁴
tBLM	QCM-D	AH peptide-tBLMs interaction involves peptide adsorption and translocation across the membrane, which could be systemically studied by varying the tethered density in tBLMs.	Appl. Mater. Today, 2020 ¹⁵⁴

SUV, Small unilamellar vesicle; SLB, supported lipid bilayer; AFM, atomic force microscopy; FRAP, fluorescence recovery after photobleaching; LUV, large unilamellar vesicle; GUV, giant unilamellar vesicle; QCM-D, quartz crystal microbalance with dissipation; (L)SPR, (localized) surface plasmon resonance; EIS, electrochemical impedance spectroscopy; tBLM, tethered bilayer lipid membrane.

*LUV is large unilamellar vesicle but it was also used in here to describe vesicles extruded through larger than 100 nm-filters. These vesicles might not only include unilamellar structures but also multilamellar ones.

2.3.2 Vesicle Suspension and High-Throughput Screening

The biophysical profiling of pharmaceutical drugs need to consider solubility, permeability, hydro/lipophilicity or biostability. Among all, model membrane system with lipid vesicle is particularly useful in permeability testing, whereby conventionally peptide-to-lipid ratio was used to compare the drug activity. There are diverse ways to utilize the vesicles by incorporating different types of probes such as ANTS/DPX fluorescence quenching, Tb³⁺/DPA complex, 6-carboxyfluorescein or calcein encapsulated within the internal aqueous compartment of the vesicle. Upon addition of membrane-permeabilizing agent, the amount of release of the encapsulated probe can be measured oftentimes-using fluorescence spectroscopy due to its wide availability and high sensitivity.

The ANTS/DPX assay involves a set of anionic dye and its cationic quencher, 8-aminonaphthalene-1,3,6 trisulfonic acid (ANTS) and *p*-xylene-bis-pyridinium bromide

(DPX), respectively (Figure 2.7A). When both molecules are entrapped inside of the vesicle, DPX quenches ANTS, thus no fluorescence signal is detected. If the pore is formed due to the addition of a membrane-permeabilizing agent, both molecules are released into the exterior of the vesicles and the ANTS will be free from the quencher, DPX. Then, the increase of fluorescence can be measured spectroscopically. Tb^{3+} /DPA assay is another binary set used in permeabilization assay. In the beginning, the cationic metal ion, terbium (Tb^{3+}), alone is entrapped in the vesicles and dipicolinic acid (DPA) in the exterior. When the membrane-permeabilizing agent is added, DPA chelates Tb^{3+} and gain fluorescence (Figure 2.7B). Both methods utilize the increased fluorescence of the system to measure the extent of membrane permeability.

By combining one of above methods and a combination of labeled lipid-quenching agent (Figure 2.7C), Krauson et al. developed orthogonal two-step assay to assess permeabilization during/after equilibrium.¹⁵⁵ Some membrane-destabilizing agents transiently permeabilize the membranes. In other words, the agents – in many cases,

peptides – initially bind to membrane and form pores, then dissipates the pore by relieving the asymmetry of the membrane breakdown.¹⁵⁶ Therefore, the leakage assessed by single dye detection can only provide information whether the pore is present at one moment (during the assay). By employing two independent probes, the active pores can be detected after the vesicle-peptide interaction has reached its equilibrium. The orthogonal assay is therefore consist of two steps: (1) initially, measure the leakage of Tb^{3+} induced by membrane-permeabilizing agent, and (2) after reaching equilibrium, add dithionite, NBD-quenching agent to check the active pores. In Figure 2.8, the example of high-throughput assay is presented. Alamethicin and LL37 were used as positive and negative control, respectively as the former one is known for a potent pore-forming peptide whereas the latter one is known not to form explicit pores. The activity based on peptide-to-lipid ratio of melittin is well known and used as a standard condition in this experiment to verify the assay setting. As expected, alamethicin showed the existence of pores in equilibrium (as can be seen by high NBD quenching ratio) and LL37 showed much lower extent (Figure XXA). Furthermore, this assay was used to screen the designed library of peptides based on melittin sequence with peptide-to-lipid ratio of 1:1000, whereby melittin is moderately effective ($\sim 50\%$ Tb^{3+} leakage and $\sim 80\%$ NBD quenching). Interestingly, only 0.1% of the library peptides showed great potency ($\sim 90\%$ Tb^{3+} leakage and $\sim 85\%$ NBD quenching), which motivated the further investigation.

Figure 2.8 The results of high-throughput orthogonal assay.¹⁵⁶ The y-axis refers to the first step, Tb^{3+} leakage, and the x-axis corresponds to the ratio of NBD-lipid quenching induced by accessible dithionite after equilibrium. Note that the lowest percentage (“No Peptide”) starts from $\sim 55\%$ as the

outer leaflet of vesicles are quenched. (A) Alamethicin, melittin, and LL37 were tested in two-step assay. The larger symbol sizes denote the higher total peptide concentrations. (B) 10,000 library compounds were tested to find out the most potent peptides derived from melittin.

Another widely used probe is fluorescein-based dyes including calcein and fluorescein-labeled molecules, which self-quench when entrapped at high concentration above 70 mM (Figure 2.7D).¹⁵⁷ This method is particularly useful as fluorescein can be easily conjugated with various sizes (*e.g.*, dextran) or functional molecules (*e.g.*, biotin-avidin). By co-encapsulating relatively large fluorescein-labeled dextran and smaller molecule (dextran¹⁵⁸ or calcein¹⁵⁹), the peptide-induced pore size within vesicles can be inferred. For example, Matsuzaki et al. reported that the melittin-induced pores have short lifetime and the pore size depends on the peptide-to-lipid ratio,¹⁵⁹ whereby another study estimated the average pore diameter is 2.5–3.0 nm.¹⁵⁸ However, highly concentrated entrapment of bulky macromolecules might be challenging and need to compensate the osmotic imbalance.¹⁰³ To address this challenge, more recently developed macromolecule-related assay utilized dextran conjugated with biotin and fluorescence probe in concentration below self-quenching.¹⁶⁰ The conjugated biotin specifically bind to streptavidin in the exterior of vesicles after the entrapped molecules were released by membrane-permeabilizing agents.

Taken together, vesicle suspension entrapped with various probes has been extensively utilized to assess the permeabilization induced by active agents in highly effective manner. High-throughput assays developed based on the vesicle leakage studies have aided in identifying novel peptide sequences that are potent. Nevertheless, the signal obtained from vesicle suspension is ensemble-averaged and the individual, specific interaction during membrane destabilization is hard to be captured. In addition, the suspension of vesicles often result in vesicle aggregation or fusion,¹⁶¹ especially when the charged peptides are present (anionic vesicle – cationic peptides).

2.3.3 Surface-Sensitive Measurements

Acoustic- and optical-based sensor techniques including ellipsometry, (localized) surface plasmon resonance (SPR), and quartz crystal microbalance with dissipation

monitoring (QCM-D) provide label-free detection at solid-liquid interfaces. Among all, QCM-D enables real-time monitoring of the adsorbed layer's mass and energy-dissipating (viscoelastic) properties, thus, it was extensively used to study binding kinetics and changes in the adsorbed layer's properties (Table 2.3). In addition, previous studies show good agreement in a correlation between biological results and the QCM-D measurements.¹¹³ In order to incorporate this acoustic-based sensor to study membrane-peptide interactions, the model platform involved planar supported lipid bilayer (SLB) and adsorbed vesicles. The main study with SLB has been conducted using artificial membrane with pure POPC and biomembrane derived from Huh7 cells, which is a human liver tumor-derived cell line often used for HCV-related studies. First, SLBs were formed on silicon oxide using vesicle fusion method, then defined concentration of AH peptide was injected to the chamber to compare the binding amount of peptide, which is indicated by the changes in the resonance frequency (Figure 2.9A).¹¹² The cell-derived membrane showed significantly higher adsorbed mass of AH peptide, suggesting that Huh7 cell-derived membranes include a specific binding receptor against AH peptide. Almost negligible effect of AH peptide within model membrane was further investigated using atomic force microscopy (AFM) and fluorescence recovery after photobleaching (FRAP), concluding that AH peptide may cause membrane thinning effect due to its amphipathicity.¹⁴⁵

Next, peptide-induced vesicle-to-bilayer formation can be tracked by QCM-D to probe the interaction kinetics. Depending on the interaction between vesicles and substrate, the adsorption of zwitterionic lipid (e.g., POPC) vesicles can result in SLBs like above (on silicon oxide) or intact adsorbed layer of vesicles (on gold or titanium oxide). QCM-D measurements enable efficient monitoring of self-assembly processes. When the intact vesicle was injected, vesicles stably adsorb on gold surface, resulting in resonance frequency (Δf) and energy dissipation (ΔD) value of -120 Hz and 7.5×10^{-6} , respectively (Figure 2.9B). This final value might vary depending on the type of vesicles such as size differences. Upon addition of AH peptide to the adsorbed vesicle, large binding of AH peptide was observed from the drop of Δf and increase of ΔD followed by reaching to the values of planar bilayer. The schematics show briefly how the QCM-D monitoring was interpreted following three steps (Figure 2.9B); (1) intact vesicle adsorption, (2) peptide binding, and (3) peptide-induced rupture and formation of SLB. Based on the results of

these studies, it should be noted that the interaction kinetic are completely distinct in SLB and intact lipid vesicle cases. AH peptide minimally and rather nonspecifically binds to the SLB platform whereas it significantly binds and induces subsequent rupture of intact vesicles.

Further characterization has revealed that AH peptide induce complete rupture of vesicles only when the average diameter is less than ~ 70 nm, whereby the pore size of extruded filter was 50 nm (as indicated in Figure 2.9C).¹¹⁵ It should be noted that the average diameter reported in QCM-D measurements does not exactly correspond to the actual cutoff-diameter of lipid vesicles that AH peptide can rupture because of two reasons; (1) the extruded vesicles show lognormal distribution in which the distribution gets broader when the extruded pore size gets larger,¹⁶² and (2) the surface-adsorbed vesicles significantly deforms due to interfacial forces.¹⁶³

Figure 2.9 QCM-D analysis performed with SLB and lipid vesicle platforms to monitor interaction between AH peptide and membranes. (A) Left: Changes in resonance frequency (Δf) starting from the formation of SLB on silicon oxide. Note that AH peptide binds significantly higher within the biomembrane derived from Huh7 cell compared to artificial membrane with pure POPC. Right: Binding mass was derived from the Sauerbrey model.¹¹² (B) Left: Intact vesicles were adsorbed on the gold surface and AH peptide was injected ~50 min. The Δf and energy dissipation (ΔD) values of -26 Hz and less than 0.5×10^{-6} , respectively, indicated the formation of SLB.¹¹⁷ Right: The schematics of AH peptide-induced vesicle rupture kinetic is depicted (not drawn to scale). (C) The vesicle size dependency of AH peptide was tested by varying the extruded vesicle size distributions

(as the nominal filter pore size is labeled). Complete rupture (last values indicating SLB) was only observed with less than 100 nm-filtered vesicles.¹¹⁵

As QCM-D detects acoustic (wet) mass, it is impossible to identify the dry mass (e.g., adsorbed layer's mass) and hydrodynamically bound solvent (e.g., aqueous water). To tackle this challenge, multiple surface-sensitive techniques based on different principles were adopted. For example, ellipsometry measures the change of polarization upon reflection or transmission, which can be converted to the optical mass or dry mass of the adsorbed materials. Taking advantage of simultaneous employment of QCM-D and ellipsometry, the wet and dry mass can be separated in each step including the peptide-mediated vesicle rupture. Then, the result can be used to calculate the peptide concentration that are bound to the membrane as well as respective peptide-to-lipid (P:L) ratio. With increasing peptide concentration from 1 μM to 31 μM , the extent of vesicle rupture increased together, indicating that a certain amount of peptide that are bound to membrane is needed for vesicle destabilization (Figure 2.10).¹⁵¹ Therefore, certain bulk peptide concentration is required to bind on the surface of lipid vesicle and reach critical density on the vesicle surface. This combined experimental approach allowed the calculation of P:L ratio, suggesting that a nearly constant P:L ratio governs the membrane destabilization and lysis. Another emerging option to combine with QCM-D is localized surface plasmon resonance (LSPR) technique, which is another optical sensor measurement with a much shorter penetration depth (10–30 nm) compared to QCM-D or SPR (100–400 nm).¹⁶⁴ A combined measurement with LSPR and QCM-D was introduced to investigate the vesicle transformation into SLB, induced by AH peptide, which revealed that the whole structural transformation is completed after the self-assembly of SLB at the close sensor surface occurs.¹⁵²

Likewise, the QCM-D technique is handy and intuitive for overviewing the whole interaction but the detailed data interpretation could be challenging when the coupled solvent is involved because careful assumptions (e.g., homogenous film properties) should be considered to model the results. On the other hand, the optical measurements such as ellipsometry and LSPR only detect dry mass, thus useful in the determination of the total mass or derived number of adsorbed vesicles, while less suited for identifying structural configurations. In addition, the vesicle rupture process is also only one part of the entire

process of structural transformation of surface-adsorbed vesicles into SLB, which is influenced by the combination of vesicle-peptide, vesicle-substrate, and (at high coverage) vesicle-vesicle interactions. Hence, structural events related to the peptide-vesicle interaction can be monitored while the ensemble-averaged kinetics being tracked represent a coalescence of individual steps within the rupture process.

Figure 2.10 The interaction of AH peptide and 50-nm extruded lipid vesicles by varied peptide concentration. (A) QCM-D Δf and ΔD monitored during the interaction of AH peptide and adsorbed vesicles. The numbers, 1 and 2, indicate vesicle and peptide addition, respectively. (B) Optical mass changes obtained by ellipsometry measurements. (C) Summary values of ellipsometry shifts. Lipid, Peptide, Peptide + Lipid refer to the adsorbed vesicle layer, additional adsorbed peptide, and the final layer after the rupture process is complete, respectively.

2.3.4 Tethered Lipid Vesicle Platform

Conventional assays are designed to characterize the ensemble-averaged changes of encapsulated probe markers from suspension of vesicles. As mentioned above in 2.3.2, the pore formation can be tracked by fluorescence probe release, resulting in dequenching in solution and subsequent increase in fluorescence emission. In these cases, the peptide-to-lipid molar ratio is commonly used to describe the activity of peptide-membrane interactions.¹⁶⁵ Although this approach can be developed as high-throughput screening, it had been pointed out that the extent of vesicle-permeabilizing potency does not always directly related to anti-infectivity in biological systems.¹⁶⁶⁻¹⁷¹ In addition, the critical peptide-to-lipid molar ratio has particularly low correlative strength to the anti-infectivity because the onset of pore formation is a necessary but insufficient step for destabilization of the lipid membrane surrounding enveloped virions.

Clarification of the mechanism of antiviral peptide has been also aided by tracking the interaction of peptides with surface-adsorbed, close-packed vesicles, which mimic the nanoscale membrane curvature of virus particles. Label-free, surface-sensitive measurement techniques can monitor the kinetics of peptide-induced vesicle rupture, leading to insights into vesicle size-selectivity,^{171, 172} surface charge preferences,¹⁴⁹ and membrane compositional effects¹⁷³ as mentioned above in 2.3.3. However, the corresponding measurements are performed on the ensemble level by monitoring net shifts in total adsorbed mass, and data interpretation is complicated by the simultaneous changes in lipid and peptide mass, which accompany stochastic events of rupture of individual vesicles. Taken together, both methods of dye leakage from vesicles in suspension and surface-based sensing measurements provide limited insight into the mechanism underlying pore formation or vesicle lysis because the collective measurements are convoluted (ensemble-averaged) and difficult to interpret as a result of peptide-induced vesicle aggregation and/or fusion.¹⁶¹ On the other hand, tethered single vesicle assay is hard to define peptide-to-lipid ratio, which limits direct comparisons of drug activity among other methods. A summary table is provided in Table 2.4.

Table 2.4 Brief summary of experimental approaches using model membrane with pros and cons.

	Bulk Vesicle Suspension	Surface-Sensing Measurements	Tethered Single Vesicle
Principle
Advantages	<ul style="list-style-type: none"> • Fast • High-throughput • Defined peptide-to-lipid ratio 	<ul style="list-style-type: none"> • Sensitive • Label-free • Real-time monitoring 	<ul style="list-style-type: none"> • Quantification of vesicle size • Real-time monitoring • Sensitive (~10 nM)
Disadvantages	<ul style="list-style-type: none"> • Ensemble-average • High lipid concentration • Vesicle-vesicle interaction 	<ul style="list-style-type: none"> • Ensemble-average • Vesicle-substrate & vesicle-vesicle interaction 	<ul style="list-style-type: none"> • Low efficiency • Hard to define peptide-to-lipid ratio

To overcome these limitations, fluorescently labeled single vesicles tethered on a passivated surface has been emerged as an excellent alternative and compatible platform to scrutinize the mechanism of both membrane pore formation and lysis mediated by membrane-active agents.¹⁷⁴ The tethering of labeled vesicles can be tracked using fluorescence microscopy techniques including confocal,^{175, 176} total internal reflection (TIR)^{177, 178} and epifluorescence¹⁷⁹ modes, whereby latter two methods enable real-time imaging of individual tethered vesicles at a single vesicle-level of high discrimination. Compared to ensemble-averaged measurements on close-packed vesicle layers, this parallel approach tracks biochemical and biophysical processes at the single-vesicle level and overcomes ensemble-averaging errors. The methodologies to immobilize vesicles without direct contact with surface have been developed over the years (Figure 2.11). Earlier passivation method varied from supported lipid bilayer (SLB) to BSA, all known to prevent adhesion of macromolecules, and the tethering strategy included biotin-avidin complex and DNA-complementary DNA strands as well as covalent bonding on gold substrate. More recent examples largely included poly(L-lysine)-grafted poly(ethylene glycol) (PLL-g-PEG) because the polymer is stable and efficient considering the sample preparation and time. The PLL backbones adsorb onto hydrophilic surface spontaneously and the grafted PEG serve as a tethering moiety.

Figure 2.11 Various tethering strategies developed from Refs. [174](#), [177](#), [180-183](#). The most common substrate was glass and passivation method included supported lipid bilayer (SLB), BSA, and PLL-g-PEG. The tethering strategy included biotin-avidin complex, DNA-complementary DNA, and covalent bonding.

Based on the individual fluorescence intensity (F.I.) of vesicle surface-embedded fluorophore, the corresponding diameter of vesicle can be derived ($\text{diameter} \propto \sqrt{\text{F.I.}}$) as established by Kunding et al.[184](#) Briefly, the extruded vesicles show size distribution represented by lognormal function or stretched Gaussian curve. The surface-embedded fluorophores represent surface area, thus the square root of F.I. can be directly proportional to the diameter of vesicle, whose distribution can be easily measured by dynamic light scattering (DLS). Depending on the fluorescence measurement strategy, different types of experiments can be performed, including those related to peptide/protein binding,

membrane permeability, and membrane fusion.^{183, 185, 186} First, protein-lipid interactions based on membrane curvature was studied by this platform, as the conventional method of bulk suspension of vesicles could not offer accurate vesicle size distribution. Hatzakis et al. first applied this knowledge and the platform to assess the membrane curvature dependent binding ability of endophilin A1 (eAH), a membrane-binding protein.¹⁸⁷ After tethering vesicles, certain concentration of labeled eAH and a control protein, streptavidin, were incubated to check the F.I. of bound proteins. As expected, the smaller vesicles, represented by lower F.I. of vesicle, recruited larger number of eAH, represented by higher F.I. (Figure 2.12A). The control experiment with the incubation of streptavidin showed no correlation with the size of vesicle (Figure 2.12B). Therefore, this study has shown the exemplary application of single tethered vesicle assay that can reveal the heterogeneous behavior.

Figure 2.12 The results obtained from single vesicle assay applied in membrane curvature sensing experiments to assess protein-lipid interaction.¹⁸⁷ (A) Surface plot of the F.I. obtained from vesicle and peptide, revealing higher F.I. from smaller vesicle (lower F.I.). (B) The protein density derived from F.I. versus vesicle diameter.

The tethered vesicles also can benefit the dual monitoring of pore formation and membrane lysis in real-time tracking of kinetics.¹⁷⁷ In Figure 2.13A, the vesicles contained headgroup-labeled, Rh-PE lipid, serving as a probe of membrane degradation whereas the encapsulated water-soluble dye, calcein, served as an indicator of pore formation. As 1 μM AH peptide was added to the tethered vesicles, the F.I. changes in Rh-PE and calcein were recorded as a function of time (Figure 2.13B). The pore formation preceded the actual membrane destabilization as monitored by Rh-PE, indicating that the membrane lysis happens after reaching a critical density of pores. In addition, extremely low concentration (~ 10 nM) of sample was utilized to monitor the changes (Figure 2.13C), which was not

possible using previously developed ensemble-averaged techniques. By relating the beginning of pore formation, onset time, and other parameters, the authors also derived the assumption that the pore might be formed by four AH peptides complex.

Figure 2.13 The results obtained from single vesicle assay applied in real-time monitoring of pore formation and membrane lysis.¹⁷⁷ (A) Schematic of the dual dye platform based on encapsulated calcein and membrane-embedded Rh-PE. (B) The real-time monitoring of F.I. during the injection of AH peptide. (C) F.I. profile of calcein based on the AH peptide concentration. (D) Histograms obtained from the release of calcein.

Another interesting application of tethered vesicle platform was to reveal the population of multilamellar vesicles by monitoring the exfoliation of each lamella by AH peptide.¹⁸⁸ As a result, the authors found that ~15% among 200 nm-filtered vesicles

demonstrate more than a single drop of F.I., reflecting the proportion of multilamellar vesicles among the population. In addition, the study also revealed that the outermost lamella of multilamellar vesicles is more susceptible to be damaged by AH peptide, in terms of pore formation and lysis, even compared to the smaller unilamellar vesicles.

Figure 2.14 The comparison of unilamellar and multilamellar vesicles.¹⁸⁸ The single drop of signals observed from small (A) and large (B) unilamellar vesicles. The double (C) and triple (D) drops were observed from the multilamellar vesicles.

More recently, Hannestad et al. combined surface sensitive measurements and single vesicle platform to show that α -synuclein (α S), a protein related to Parkinson's disease, promote membrane destabilization based on lipid specific interactions.¹⁸⁹ Most importantly, the single vesicle assay was monitored not only by fluorescence microscopy but also with scattering microscopy. First, surface-sensing techniques, QCM-D and SPR, were used to demonstrate the binding of α S onto vesicles, made from DOPG and DOPS, whereby both are anionic lipids from mitochondrial and synaptic membranes, respectively. In Figure 2.15A, the QCM-D result shows that α S significantly bind to DOPS rather than DOPG, followed by slight increase of resonance frequency, indicating structural transformation. Next, by the combination of label-free scattering and fluorescence microscopy, an interesting observation was noted in DOPG case that the changes induced by α S in the fluorescence signal were small while the scattering intensity showed significant reduction of the vast majority of vesicles (Figures 2.15B and C). The authors interpret this observation

was due to a sudden asymmetric collapse of vesicle structure, leaving behind the lipid materials.

Figure 2.15 The combination of surface sensitive measurements and single vesicle platform to assess the interaction between anionic lipids, DOPG and DOPS, and αS .¹⁸⁹ (A) QCM-D measurements show the binding of αS more significantly to DOPS vesicles. (B) Scattering and fluorescence micrographs of tethered vesicles before (t_1) and after (t_2) the addition of αS . (C) The fraction of vesicles that are visible in scattering microscopy and the ratio between before and after the addition of αS .

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Chapter 3* Experimental Methodology

This chapter describes the experimental methodology used in the thesis, including the fabrication of various lipid model membrane platform and surface measurement techniques. First, the rationale selection of experimental approach is introduced, followed by an overview of the materials that are used in the projects. Then, the rest of the chapter highlights the principles and description of each experimental techniques to study antiviral agents, including secondary structure in solutions and interactions with various types of model membranes including vesicles and planar lipid bilayers. Importantly, the details of fabrication of model membranes and corresponding characterization methods are emphasized to introduce the application of tethered lipid vesicle platform, which models viral membrane and study the antiviral agents' activities with diverse surface sensing measurement techniques and fluorescence microscopy.

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3.1 Rationale for Selection

In *Chapter 3*, the experimental methodology used in this thesis is detailed in three sections: materials, preparation of samples, and experimental techniques. A key motivation behind the selection of experimental strategies in this thesis is to establish non-clinical antiviral drugs testing platform to select the potential broad-spectrum antiviral drugs to be tested in pre-clinical or clinical stages. Antiviral drugs testing is difficult because of three main reasons. First, many clinically important viruses are difficult or impossible to cultivate in *in vitro* settings.¹ Second, there is no perfect animal model for therapeutics research so that multiple models should be tailored and tested (meaning that multiple animal experiments are needed).² Lastly, it is simply dangerous to use virulent viruses. Despite these difficulties, most studies were done based on such biological readouts or viral growth inhibition. While such approaches aided the evaluation of biological activities, it remains elusive to characterize and explain underlying mechanistic details. Moreover, there is a limitation in improving the strategies to control the drug's activity, which can be resolved if there is a platform to real-time monitor the membrane-drug interaction.

The lipid envelope antiviral disruption (LEAD) strategy directly target the viral envelope and cause membrane destabilization, resulting in virus particle lysis and loss of infectivity.^{3, 4} Therefore, LEAD can be non-clinically tested by lipid model membranes mimicking viral envelopes, which are cost-friendly, safe, and most importantly, providing an opportunity to tune the membrane destabilization activity by engineering peptide sequences. One of the most important criteria of antiviral agents is to be selective toward virus particles and not to be harmful toward the host cells or machineries. Amphipathic α -helical (AH) peptide is found out to be selective by sensing the membrane curvature, which is particularly effective when the viral particles are small enough (<100 nm) considering that the usual host cell membranes (10–100 μ m) are ~1000-fold larger. This size discrimination can be tested using various sizes and shapes of lipid model membranes. Taken together, this section will discuss the principles underlying the platform design, characterization, and modeling techniques employed and the methods of data analysis, including errors.

The materials and preparation of samples will mainly involve amphipathic peptides, which are the lead antiviral drug candidates and lipid reagents used in the fabrication of model lipid membranes. The lead sequence, AH peptide, is derived from the N-terminus of the nonstructural protein (NS5A) of Hepatitis C virus (HCV).⁵ For lipid agents, unsaturated phospholipids and small fractions of functional lipids (fluorophore, charges, or sterols) will be introduced. These lipid molecules will be self-assembled into several types of model membranes, high-curvature-membranes of viral envelopes were modeled by small unilamellar vesicles and low-curvature-membranes such as host cell membranes were mimicked by tethered planar lipid membranes. In the experimental section, the first half is consist of the techniques to characterize biophysical aspects of peptide, starting from the concentration measurement and to secondary structure analysis, which were measured by ultraviolet-visible (UV) spectroscopy and circular dichroism (CD) spectroscopy, respectively. Then, all experimental techniques and their principles are described in the final section. The interaction between peptides and model viral membrane, including small vesicles, clusters of vesicles, and planar lipid membranes will be evaluated using multiple surface-sensitive measurement techniques.

3.2 Materials

3.2.1. Amphipathic α -Helical (AH) Peptide

The high-purity peptides used in this thesis were synthesized by AnaSpec Corporation (Fremont, CA, USA) using F-moc solid-phase synthesis. The purity was measured by high-performance liquid chromatography using reverse-phase, resulting in 96% and 95% for AH and C5A peptides, respectively. The lyophilized peptide powder was packaged in 2 mg individual vials and stored in -20°C freezer before the usage. Then, AH and C5A peptides were solubilized in deionized water to prepare a highly concentrated stock solution of 2 mg/mL. Before each experiment, the molar concentrations of peptides were determined by measuring the UV absorbance at 280 nm wavelength. The ratio between the mass value obtained from this molarity determination (calculated mass based on molecular weight) and the weighed mass is $>90\%$ and supports that the peptides have

Blue Letter: Conservative mutation
Red Letter: Non-conservative mutation

Conserved residues:

Acidic		Basic
	Hydrophobic		Thiol

Note: All sequences start with amino group (N-terminus) and end with carboxyl group (C-terminus). In case of C-terminal amidation, isoelectric point (pI) is ~2.4 higher than carboxylation.

3.2.2. Lipid Reagents

66

Figure 3.2 Molecular structures of the tested lipid that are used for the thesis.

3.2.3. Buffer Reagents

Tris buffer solution [10 mM Tris (pH 7.5), 150 mM NaCl] was used in this thesis to fabricate vesicles or bilayers, or dilute peptide samples unless otherwise specified (*e.g.*, circular dichroism study in 100% deionized water or 50% trifluoroethanol). The Tris buffer is based on tris(hydroxymethyl)aminomethane (Tris), which contains a primary amine that can function as a buffer ($pK_a=8.1$). Tris and sodium chloride was purchased from VWR (Singapore) and Merck (Singapore), respectively. First, 1.21 g of Tris and 8.76 g of sodium chloride were dissolved in 1 L of deionized water (Milli-Q-treated water ($>18\text{ M}\Omega\cdot\text{cm}$), Millipore, Billerica, MA) using magnetic stirrer. High concentration ($>5\text{ M}$) of hydrochloric acid (HCl) was used to adjust the solution pH to 7.5.

3.3 Preparation of Samples

3.3.1. Preparation of Peptide Solutions

The lyophilized peptide samples were kept at -20°C for long-term storage in 2 mg individual vials. A vial containing 2 mg was solubilized in 1 mL deionized water to prepare a highly concentrated stock solution of 2 mg/mL. In all peptide samples used in this thesis was water-soluble and organic solvents were not used. Then, the sample was mixed by gentle pipetting and was not subjected to vortexing. After carefully checking that the peptide solution is transparent and well-dissolved, the molar concentrations of peptides were measured by the UV absorbance at 280 nm wavelength. AH peptide typically resulted in $\sim 600\text{ }\mu\text{M}$, which is $\sim 98\%$ of calculated mass based on 2 mg/mL. The ratio between the mass value obtained from this molarity determination (calculated mass based on molecular weight) and the weighed mass is $>90\%$ and supports that the peptides have high molarity. Then, the stock solution was distributed in 0.2 mL microtubes in the amount of one-time usage and stored at -20°C until the next experiment. The thawed sample was further diluted in Tris buffer to obtain the desired molar concentration immediately before experiment. The remained stock solution was kept in a fridge (4°C) for other experiments in the same day and discarded after a day.

3.3.2 Tethered Lipid Vesicle Preparation

3.3.2.1. Vesicle Extrusion

The lipid vesicles or liposomes are enclosed lipid bilayers that can be commonly found within or outside of cells. Especially in this thesis, lipid vesicles serve as a model of viral envelope, thus controlling the properties (*e.g.*, size and distribution) of vesicles are critical in the experimental results. Among several methods such as Bingham, sonication, or ethanol injection, the extrusion method is the most widely used and extensively studied as well as its principle is quite straight-forward. The vesicle extrusion method involves forcing lipid suspension through membrane pores so that (multilamellar) vesicles break down into relatively monodisperse and smaller (and mostly unilamellar in the case of pore size $<100\text{ nm}$) vesicles closer to the pore size. The experimental parameters such as pore size,⁷ the number of times the suspension went through,⁸ temperature,⁹ pressure,^{10, 11} and lipid compositions¹² that affect the size distribution of vesicles are widely reported. Although the extrusion method requires trained and experienced technique, it is simple,

does not require organic solvents, is easy to manipulate sizes, produces highly reproducible size distribution, relatively rapid, and applicable to a wide range of lipid compositions.

In this thesis, all vesicles were prepared by extrusion method. First, the desired lipid compositions were dissolved and mixed in chloroform solution together, then dried under a stream of nitrogen at the room temperature. For tethered lipid vesicle experiments, phospholipid and/or sterol mixture included 0.7 mol% of Rh-PE (maximum excitation and emission wavelengths of 560 and 583 nm, respectively) and 0.1 mol% DSPE-PEG(2000)biotin. The overall lipid concentration was maintained to be 2 mg/mL. Then, the dried lipid film was placed under vacuum for at least 2 h to remove residual chloroform. It should be noted that more than 12 h of storage of dried lipid film in desiccator is also not desirable especially when the equipment is shared. Afterwards, the extruder set (Avanti Polar Lipids, Alabaster, AL) including three different syringes, filter supports (two pieces in each side), polycarbonate membranes with nominal pore sizes (50, 100, or 200 nm), outer casing of extruder, retainer nut, two internal membrane supports, and Teflon bearing were assembled (Fig. 3.3A).

After setting up the extrusion kit, dried lipid films were hydrated in Tris buffer with 14.3 mM calcein dye (maximum excitation and emission properties of 494 and 517 nm, respectively, which is well-suited for tracking applications¹³) to facilitate dye encapsulation within the lipid vesicles. During the hydration, the lipid molecules self-assemble to form multilamellar phases, which scatter light, thus the resulted suspension was opaque. Afterwards, to increase the efficiency of entrapment of water-soluble compounds or to decrease the vesicle size distribution and multilamellarity, the lipid suspension was subjected to five freeze-thaw cycles by alternately plunging the vial of lipid suspension in a liquid nitrogen bath and 60 °C water bath followed by vortexing. Then, the sample was loaded into a gas-tight syringes prepared for the extruder and placed into the extruder kit. In order to minimize the dead volume, the interior of syringe as well as filter/supports can be pre-wet by passing through water or buffer. Meanwhile, another empty syringe set to zero was placed into the other end of the extruder. After setting up the fully assembled extruder apparatus (Fig. 3.3B), the filled syringe was pushed (with constant force) until the lipid suspension is almost completely transferred to another syringe. Then, the alternate syringe was pushed to transfer the lipid suspension to the original syringe. This cycle was

repeated for 10 times and in the last step, the lipid suspension was left in the alternate syringe, not the original one. This means that the lipid suspension passed through the membrane for 21 times. The higher number of cycles through the membrane generally will produce the more homogenous vesicles. In order to minimize the contamination, last five cycles were performed after replacing the original syringe to the third clean syringe. Then, the final extrusion was filled in the third alternate syringe. The resulted vesicle solution was stored at 4°C fridge for further usage. It should be noted that the small vesicle solutions are not stable in aqueous media for more than 3–4 days. Then, the whole apparatus was cleaned thoroughly with ethanol and water and dried.

Figure 3.3 Avanti Mini-Extruder internal schematic (A) and fully assembled extruder apparatus (B).

3.3.2.2. Microfluidic Chamber Assembly

To enable real-time monitoring using inverted microscopy, microfluidic setup was prepared. First, the glass coverslip (25 mm × 75 mm, thickness 170 ± 5 μm, ibidi GmbH, Martinsried, Germany) was rinsed with sodium dodecyl sulfate (SDS) 1 w/v% solution, deionized (DI) water, and 95% ethanol subsequently. Then, the substrate was dried with nitrogen gas, then was subjected to oxygen plasma (Harrick Plasma, USA) for at least 30 sec to treat the surface hydrophilic and remove residual contaminants or impurities from the surfaces. Shortly after the plasma treatment, the glass coverslip was assembled into the microfluidic chamber (sticky-Slide VI 0.4, ibidi GmbH or home-built) while ensuring the plasma-treated surface faces the adhesive part of the fluidic chamber (Fig. 3.4). Right after the assembly, the channels were immersed in poly(L-lysine)-grafted poly(ethylene glycol)-

biotin (PLL-g-PEG(-biotin) 50%, SuSoS AG, Switzerland, 41.5 $\mu\text{g}/\text{mL}$ diluted with the Tris buffer) solution for at least 30 min. During this process, the oxide surface allows positively charged poly-L-lysine (PLL) backbones to adsorb spontaneously. Of note, the coating step with PLL-g-PEG(-biotin) can proceed the assembly of microfluidic chamber (right after plasma treatment). Then, the surface was incubated with 10 $\mu\text{g}/\text{mL}$ neutravidin for at least 2 min, followed by rinsing with the buffer.

Figure 3.4 An optical image of the assembled home-built microfluidic chamber indicating the inlet and outlet with a schematic showing the internal chamber.

3.3.2.3. Tethered Vesicle Platform

A prerequisite for using dye-loaded vesicles is to separate the free (not encapsulated) dyes from the (encapsulated) vesicles. One of the widely used tool to separate small molecules from large vesicles is size-exclusion chromatography or gel filtration.¹⁴ In this thesis, Sephadex G-25 gel filtration column (GE Healthcare Life Sciences, Pittsburgh, PA, USA) was chosen to filter calcein-loaded vesicles. Sephadex is a crosslinked dextran gel, whereby the porosity is controlled by the degree of crosslinking.¹⁵ During the aqueous sample (vesicles and calcein in Tris buffer) going through the gel column, large molecules (vesicles) are excluded while smaller molecules (free calcein) enter the pores (Fig. 3.5A). As a result, large molecules elude first, followed by smaller molecules (Fig. 3.5B).¹⁶ After a stable setup of the gel column, 2 mL of deionized water was passed through the column, followed by 1.5 mL of Tris buffer. Right before Tris buffer reached to the upper end of

column, 1 mL of 10-fold diluted (0.2 mg/mL) vesicle sample was injected. When the vesicle sample reached the upper end of column, 0.5 mL of Tris buffer was injected two times sequentially before the medium ran out and eluted solution was collected in 2 mL tube.

Figure 3.5 Size exclusion chromatography (gel filtration) for lipid vesicle purification. (A) Principle of gel filtration. (B) Vesicle purification containing free calcein on a gel column.¹⁶ Vesicles are collected first (earlier fraction number) because the size of them are bigger than free calcein molecules.¹⁶

Next, the tubings that connect to the vial of sample and peristaltic pump were assembled to the microfluidic chamber, then it was mounted on the sample stage. The prepared vesicles were injected in a flow rate of 100 $\mu\text{L}/\text{min}$ and it was monitored by epifluorescence microscopy. When the biotinylated vesicles non-covalently, but strongly immobilized onto the coverslip surface by biotin-neutravidin chemistry (Fig. 3.6A), the focal plane was adjusted to carefully monitor the amount of vesicles attached. After reaching ~ 100 – 200 vesicles per a field of view, the chamber was rinsed with Tris buffer (Fig. 3.7B). Of note, the removal of unattached vesicles should be conducted thoroughly in order to avoid disruptions caused by floating vesicles during the monitoring.

Figure 3.6 The schematic (A) and observation using epifluorescence microscopy in red (Rh-PE) and green (calcein) channel (B) of tethered vesicle platform.

3.3.3 Tethered Bilayer Lipid Membrane Preparation

Tethered bilayer lipid membranes (tBLMs) are planar lipid bilayer structures, whereby the inner leaflet is partially or fully associated with anchor or tether molecules.¹⁷⁻¹⁹ This platform is particularly useful to monitor the membrane permeability or to incorporate transmembrane proteins compared to conventional supported lipid membranes.²⁰ The fabrication of a tBLM generally involves surface modification of the support substrate with a monolayer of covalently-attached tether molecules prior to lipid bilayer deposition.¹⁷⁻¹⁹ These tether molecules typically present surface modifying functional groups at one end (e.g., thiol/disulfide groups for attachment to gold,²¹ silane groups for attachment to silicon oxide²²) and a hydrophobic segment at the opposite end to accommodate the inner leaflet of the lipid bilayer. A sparsely tethered bilayers is composed of not only tether molecules but also spacer molecules, which can compete with the tether moieties during the surface modification step, resulting in an increased lateral space between the tethering molecules.¹⁹ In this thesis, sparsely tethered membranes were used to examine the membrane permeability of antiviral peptides using electrochemical impedance spectroscopy (EIS).²³⁻²⁵

The tBLM kit was manufactured by SDx Tethered Membranes (Roseville, New South Wales, Australia). The kit included devices such as membrane conductivity reader and electrode flow cell cartridge, as well as lipid agents including pre-coated slides. The

fabrication of tBLMs has done in three steps as follows; (1) the deposition of tether and spacer molecules on the gold support substrate to form a covalently attached self-assembled monolayers, (2) the incubation of mobile lipids dissolved in an organic solvent over the self-assembled monolayers and (3) rapid rinsing with buffer to self-assemble the tBLM (Figure 3.7A).²⁶ Both tether and spacer molecules possess benzyl disulfide groups, which form sulfur-gold covalent bonds with the gold electrode (Fig. 3.7B). Each tether molecule consists of eight oxygen-ethylene-glycol group terminated by a C20 phytanyl chain, which will be later incorporated into the inner leaflet of the tBLM. On the other hand, each spacer molecule consists of four oxygen-ethylene-glycol group terminated by a hydroxyl group to ensure the overall hydrophilic integrity of the membrane platform. The half-length is useful in increasing the volume of the sub-membranous aqueous reservoir, which does not directly interact with the membrane, but allows other membrane-active molecules (e.g., peptides, proteins) to be inserted into bilayer patches (Fig. 3.7C).

Figure 3.7 Schematic illustrations of the tethered bilayer lipid membrane (tBLM) used in this thesis. (A) The sequential steps involved in the formation of tBLMs on the gold surface. (B) The structure of the tether and spacer molecules with hydrophobic, hydrophilic, and disulfide residues shaded in grey, red, and yellow, respectively. (C) The formation of DMPC tBLMs using the tether and spacer molecules presented in (B). The figure is adapted from Ref. ²⁶.

The SDx gold electrodes were pre-coated with 10% tether molecules and 90% spacer molecules (T10) and stored in ethanol solution at 4 °C (step 1). Therefore, one should note that the gold electrodes were fully soaked in ethanol while opening the package. Then, the electrodes were partially dried at room temperature to remove the excess ethanol before assemble them into a flow cell cartridge (tethaplate), which is composed of white polycarbonate flow cell and a slide with pre-coated six gold electrodes. Then, 8 μ L of mobile lipid solution in ethanol (in this thesis, DOPC) was added to the flow cell in $\sim$ 20 s intervals to fill all six electrodes (step 2). After finishing the loading of the sixth cell, 200 μ L of Tris buffer solution was added to the first cell to promote bilayer formation by

exchanging the organic solvent to aqueous solution (step 3). This step was repeated for two more times (total 600 μL of buffer was used) to rinse the excess lipids. Then, the cartilage was plugged in to the membrane conductivity reader (tethaPOD). The initial conductivity and capacity values should be less than 2 μS and around 15–25 nF, respectively, which indicate good sealing properties of tBLM.²⁷

3.4 Experimental Techniques

3.4.1 Peptide Concentration Measurement

The quantification of peptide concentration is an important step before evaluating any type of interaction or activity of peptide. The actual peptide concentration derived from its apparent weight (dry mass weighed by weighing scale) might be inaccurate because of bound water, salts or counterions, thus ultraviolet (UV) absorbance measurements by side chains of amino acids were mainly used in this thesis. The absorbance of UV light at 280 nm can be used when tryptophan and tyrosine residues are present in the sequence^{28, 29} as AH and C5A peptides include 4 and 3 tryptophan residues, respectively. As buffer components might interfere the readouts, the stock solution of peptide solution was first prepared in pure deionized water. Also, the lyophilized peptide samples were already tested using HPLC that their purity ranges are over 96%, which increases the reliability of the amino acid analysis since any impurities that can contribute to the absorbance at 280 nm interferes the result.

In UV-visible spectroscopy, the concentration is calculated by Beer-Lambert Law:

$$A = \epsilon cl$$

where A is absorbance, ϵ is extinction coefficient, c is molar concentration of peptide sample, and l is optical path of quartz cuvette in centimeter. Generally, the path length of cuvette is 1 cm as well as in this thesis. The molar extinction coefficient was calculated as

$$\epsilon_{280} = a\epsilon_{tyr} + b\epsilon_{trp} + c\epsilon_{cys}$$

where a , b , and c are the number of each type of amino acid residue per peptide molecule, ϵ_{tyr} is 1280/ $\text{M} \cdot \text{cm}$, ϵ_{trp} is 5690/ $\text{M} \cdot \text{cm}$, and ϵ_{cys} is 120/ $\text{M} \cdot \text{cm}$. Therefore, the molar

extinction coefficient of AH and C5A peptides were calculated as 22760 and 17070 per M · cm, respectively.

The UV spectroscopy and quartz cuvette used in this thesis were Boeco-S220 spectrophotometer (Boeco, Hamburg, Germany) and Hellma micro-cuvette (Hellma GmbH, Müllheim, Germany, cat. no. 108-002-10-40), with 1 cm light path, respectively. First, the baseline was set by using 500 uL deionized water as a blank solution at 280 nm. Then, 10 uL from the blank solution was taken out from the cuvette and 10 uL of peptide stock solution (usually prepared in 2 mg/mL) was refilled to make 50-fold diluted peptide sample solution. By using 200 uL pipette, the solution in cuvette should be mixed well. Then, the concentration of peptide was measured at 280 nm, whereby the absorbance should range in between 0.1–1.0. Typically, when the stock solution is prepared with 2 mg/mL, the absorbance readout is around 0.2–0.3, which is when the accuracy is the greatest.²⁸ Then, the peptide concentration is calculated by Beer-Lambert Law.

3.4.2 Secondary Structure of Peptides

The secondary structure of peptide is three dimensional structure that can be commonly categorized as helices, beta (β) sheets, turns, loops, or random coils (absence of regularity), whereby the most common structures are α -helices and β -sheets. The peptide structure is determined by the patterns of hydrogen bonding and backbone conformation, which provides insights into how peptides achieve their functions (*e.g.*, membrane pore formation) and interact with other ligands, lipids, or proteins.³⁰ One of the most sensitive techniques to study secondary structures are X-ray crystallography³¹ or NMR spectroscopy³² but the prerequisite of sample preparation (*e.g.*, certain solution, high purity, diffraction quality crystals) limits its utility. As for a routinely performed and well-established technique is circular dichroism (CD) spectroscopy, which records the difference in absorbance of left- or right-handed circularly polarized light as a function of wavelength (UV range) aroused from the asymmetry of molecules. Most of amino acids are chiral (mirror-image) molecules, which are not superposed by any rotations or translations, thus CD spectroscopy is broadly used to study peptides or proteins in solution-based environment with various conditions of solution (buffer, salts, ligands) or

temperature. Also, rapid data collection and requirement of small amount of sample are other reasons why CD measurement is selected in this thesis and many others.

The CD spectroscopy used in the thesis was Chirascan spectropolarimeter (Model 420, AVIV Biomedical Inc.) with a 0.1 cm path length cuvette (Hellma). Solvents including Tris buffer or 50% 2,2,2-trifluoroethanol (TFE, Sigma-Aldrich, St. Louis, MO, USA) in deionized (DI) water were always freshly prepared to avoid any contamination or inaccuracy caused by evaporation (TFE is highly volatile) and the concentration was always measured right before conducting CD spectroscopy. All peptide concentrations were prepared to 100 μ M by diluting aliquots with respective solvents. First, the baseline spectra using respective solvents were collected to later subtract the CD spectra of samples. Spectra data were collected in triplicate at 25 °C, wavelengths ranging from 190 to 260 nm with 1 nm bandwidth with a step size of 0.5 nm and time constant of 0.1 s. The final averaged spectra were calculated in mean residue molar ellipticity:

$$[\theta] = \frac{\theta}{10 \times c \times l}$$

where θ is the ellipticity, c is the molar concentration measured by UV spectra (100 μ M in most of the cases), and l is the path length in centimeter (0.1 cm). The presented spectra were smoothed by the Savitzky-Golay method with a polynomial order of two. The fractional helicity (fH) of the peptides was calculated as follows

$$fH = \frac{[\theta]_{222}^{obs} - 3000}{-36000 - 3000}$$

where $[\theta]_{222}^{obs}$ is the molar ellipticity at 222 nm.^{33, 34}

3.4.3 Dynamic Light Scattering (DLS)

The size distribution profile of lipid vesicles prepared by extrusion can be characterized by cryogenic transmission electron microscopy (cryo-TEM), static (SLS) or dynamic light scattering (DLS). Among them, light scattering methods are the most commonly used technique over others due to relatively simple sample preparation and noninvasive characteristic.^{35, 36} The principle of DLS is based on Tyndall effect (light scattering) of small particles in solutions that are undergoing Brownian motion.³⁷ In Tyndall effect, the intensity of the scattered light is inversely proportional to the fourth power of the

wavelength and the sixth power of its size, which fluctuates over time because of random diffusion of particles, namely Brownian motion. Combining these two principles, DLS measurement analyzes the time-dependent fluctuations of the scattering light intensity coming from the continuously diffusive movement of small particles within dispersion. To analyze the experimental light scattering data, the fluctuation of scattering light intensity is correlated against short decay time intervals (τ). When the measurement time goes on, the location of the initial particle will be far away from the time when it is measured. Then, the intensity autocorrelation function, $g(\tau)$, which describes how the given light scattering measurement relates to itself in a time dependent manner, is calculated. For example, when the time is set to 0, the $g(\tau)$ will be 1 whereas the time is infinite, the $g(\tau)$ will be 0, where there is no more existing correlation. The rates of the fluctuations were determined through the autocorrelation analysis based on the Stokes-Einstein equation, which is relating the particle size to its diffusion coefficient:

$$D = \frac{k_B T}{6\pi\eta R}$$

where D is diffusion coefficient, k_B is Boltzmann's constant, T is the absolute temperature, η is the viscosity of solution, and R is the hydrodynamic radius of the spherical particle. Therefore, the signal of larger particles will change slowly (the correlation exists for a longer period of time) compared to smaller particles.

Typically, including this thesis, the size of vesicles were analyzed in the term of an average vesicle size and polydispersity. The diameter measured by DLS can be defined in three different ways; number, volume, and intensity distribution. The abovementioned autocorrelation results in the intensity size distribution, then volume and number distribution are calculated based on specific assumptions. Therefore, the most direct, reproducible, and the most relevant representation is generally regarded as the intensity size distribution because the volume or number distribution might be deviated from the reality when the assumptions inherent in the transformation are not the case. The number distribution shows the number of particles, which can be simply expressed as

$$\%N_a = \frac{100 \cdot N_a}{N_a + N_b + \dots + N_x}$$

where $\%N_a$ is the relative number of the particle with size “ a ”. Then, assuming that the

particles are fully spherical, the volume of particle is proportional to the size to the third power. Therefore, the volume distribution is represented as

$$\%V_a = \frac{100 \cdot N_a \cdot a^3}{N_a \cdot a^3 + N_b \cdot b^3 + \dots + N_x \cdot x^3}$$

where $\%V_a$ is the relative volume of the particle with size “ a ”. Finally, assuming that the scattered light intensity of a small, isotropic, spherical particle is proportional to the size to the sixth power (Rayleigh scattering), the intensity distribution is described as

$$\%I_a = \frac{100 \cdot N_a \cdot a^6}{N_a \cdot a^6 + N_b \cdot b^6 + \dots + N_x \cdot x^6}$$

where $\%I_a$ is the relative amount of light intensity of the particle with size “ a ”. Taken together, the assumptions underlying the derivation from the intensity distribution to volume or number distribution are the shape (spherical) and the optical properties (*e.g.*, Rayleigh scattering) of particles. As extruded vesicles show lognormal size distribution, the intensity-weighted size distribution was used in this thesis to directly compare the different sizes of vesicles by extruded pore sizes (Fig. 3.8) or to acquire the relationship between fluorescence light intensity and vesicle sizes.

The DLS used in this thesis was a ZetaPals particle size analyzer (Brookhaven Instruments, Holtsville, NY) with the scattering light angle of 90° to minimize the reflection effect. All autocorrelation functions resulted were analyzed by the cumulants method and fitted to a lognormal destitution to obtain the vesicle size distribution. The disposable cuvette was purchased from Sigma Aldrich (St. Louis, MO) and used right after opening the seal. The vesicle samples were diluted in a $\sim 10:1$ in volumetric ratio with Tris buffer solution. Of note, the polydispersity was ensured to be less than 0.1 in most of the cases except for larger filter pore sizes than 200 nm and the average count rate ranged 100 to 450 kcps. The sample quality baseline was close to 100% and the each sample was measured by 5 min in 1 min intervals. If the measurements were out of these ranges, the samples were diluted again in a new cuvette to ensure contaminants as very small amount of dust might result in a huge error.

Figure 3.8 Size distribution of vesicles determined by DLS. The intensity-weighted DLS data shows the size distribution (reported as hydrodynamic diameter) for vesicle populations with different filter sizes of 50, 100 and 200 nm.

3.4.4 Fluorescence Microscopy

The tethering of single vesicle can be tracked using fluorescence microscopy techniques including confocal,^{38,39} total internal reflection (TIR)^{40,41} and epifluorescence⁴² modes by incorporating fluorescence dye molecules. Particularly, time-lapsed imaging ability of fluorescence microscopy enables parallel measurements including thousands of lipid vesicles in a single experiment. Depending on the measurement strategy, the general type of fluorescence microscopy is classified into three; TIRF, confocal, and epifluorescence microscopy (Figure 3.9). The total internal reflection occurs when the incident light angle is greater than the critical angle, generating an evanescent wave, which

is a thin electromagnetic field that propagates parallel to the interface and excites the fluorescent molecules. Therefore, TIRF can obtain a wide-field imaging without interference aroused from the non-focal plane. However, the evanescent wave occurs in the depth of 50–250 nm adjacent to the coverslip, which might be a limiting factor during the imaging of large sizes of vesicles (>600 nm). Confocal microscopy utilizes a pinhole in front of the photodetector to filter the signals from non-focal plane. However, the rate of image acquisition is limited because the images are constructed by sequential point signals obtained during the scanning of the sample. The illumination in epifluorescence microscopy targets an entire sample and the emission throughout the entire volume can be detected. Therefore, there is a direct proportionality between the integrated intensities and the number of fluorophore labels on the surface.⁴³ As fluorophores are evenly distributed along the surface and vesicles, the integrated intensity can be related to the actual vesicle size (diameter).

Figure 3.9 Fluorescence-based microscopy techniques. From left, TIRF (total internal reflection fluorescence) microscopy, confocal microscopy, and epifluorescence microscopy are presented.

As single vesicle assay involves large amount of data in a single measurement (*e.g.*, 200 vesicles per field of view $\times$ 8 different locations $\times$ 100 time points), computational approach is used to analyze the images. In this thesis, the single vesicle analysis was performed with the aid of Python(x,y) software to derive valid outcome by approaching statistically with thousands of individual vesicle, which require least mean square problem, morphological operation and linear/nonlinear fitting strategies. The whole process was composed of three steps of background removal, fluorescence intensity extraction by labeling individual vesicle, and linear or non-linear fitting to acquire histogram (Figure 3.10).

Figure 3.10 An overview scheme of the data analysis process of single vesicle platform by Python.

In the background correction stage, the least square problem (LSP) is employed to minimize the sum of squared residual errors $f(x) = \frac{1}{2} \sum_i r_i(x)^2$. We can define minimizing problem using any other norm, like minimizing first order norm $\|r_i\|_1 = \sum_i |r_i|$ or infinity norm $\|r_i\|_\infty = \max_i r_i$ instead of the second order norm. But, the second order norm can be derivative, thus, it is easier to be formulated than others. The second order Taylor expansion of the error function at the optimum point x^* is

$$f(x^* + \delta) = f(x^*) + \nabla f(x^*) \cdot \delta + \frac{1}{2} \delta^T \cdot \nabla^2 f(x^*) \cdot \delta + O(\delta^3)$$

The gradient $\nabla f(x)$ and Hessian $\nabla^2 f(x)$ are

$$\nabla f(x) = \sum_i r_i(x) \nabla r_i(x) = J^T(x) r(x)$$

$$H(x) = \nabla^2 f(x) = \sum_i \nabla r_i(x) \nabla r_i(x)^T + \sum_i r_i(x) \nabla^2 r_i(x) = J(x)^T J(x) + \sum_i r_i(x) \nabla^2 r_i(x)$$

Where Jacobian is

$$J(x) = \left[\frac{\partial r_i}{\partial x} \right] = [\nabla r_1(x)^T, \nabla r_2(x)^T, \dots, \nabla r_m(x)^T]^T.$$

The first derivative of $f(x)$ by δ should be zero because it is optimum in

$$\frac{\partial f(x^*)}{\partial \delta} = \nabla f(x^*) + \nabla^2 f(x^*) \cdot \delta = J^T \cdot r(x^*) + H(x^*) \cdot \delta = 0.$$

The linear least square problem solves the problem, in which the residual has a linear relation, $\nabla^2 f(r_i(x)) = 0$. The comparison of Python correction intensity value and manual subtraction is shown in Figure 3.11A. The discrepancies between two values are there, but the limitation of manual subtraction by ImageJ software should be considered. It is difficult to get a consistent result because the background selection can be varied in the size of extent and the location (how close to the neighbor vesicles). Most of all, it is almost impossible to manually select backgrounds for thousands of vesicles. Then, individual vesicle's fluorescence intensity is extracted by labeling all vesicles over the threshold that has been given. Combined Excel file is exported in a separate folder and additionally, individual fitting graphs are provided such as Figure 3.11B. After overviewing the trends, gradients and lag time can be calculated to obtain histograms. Gradients can be used in the buffer experiment to evaluate the leakage and bleaching of the dye. If the calcein leaks fast, the absolute value of gradient increases. Lag time is used when the peptide interacts with vesicles to rupture or make pores.

Figure 3.11 (A) The comparison of manual subtraction of background intensity and Python correction. (B) Output after the second step of vesicle intensity extraction.

The representative result of single vesicle platform is presented in Figure 3.12. AH peptide is known to rupture small-sized vesicles ($< \sim 120$ nm) in previous studies based on surface-based assays such as QCM-D.⁴⁴ Extruded vesicles had an average diameter of 122

nm (data not presented), as determined by dynamic light scattering (DLS). In the experiments, 10, 100, and 1000 nM of AH peptide was injected to the tethered vesicles, and the drop in rhodamine and calcein signals were observed as a function of time. In 100 and 1000 nM of AH peptide, all vesicles were ruptured within 20 min by showing discrete two steps of kinetic in both rhodamine (vesicle lysis) and calcein (pore formation) signal independent of vesicle size, as presented in representative traces of individual vesicles (Figure 3.12A). In lower concentration of 10 nM, two different types of kinetic was observed depending on the different sizes of vesicles. The small vesicle is degraded entirely by showing final normalized intensity less than 0.1 whereas the big vesicle remains after 60 min by showing a slow decrease in fluorescence intensity. Also, the efficiency can be obtained according to vesicle's size. The sizes ranging 25 to 75 nm showed 97.1% rupture (final normalized intensity < 0.2), then the ratio of ruptured vesicle decrease significantly with the increase of vesicle size.

Figure 3.12 Examples of data representation of single vesicle assay. (A) 100 nM and 1000 nM of AH peptide were tested to monitor pore formation (calcein) and membrane lysis (rhodamine). A single-step drop in fluorescence intensity is observed in both calcein and rhodamine. Insets are histograms of the time corresponding to the onset of pore formation or rupture obtained from >500 individual vesicles. (B) 10 nM of AH peptide was tested to evaluate vesicle size dependency. Left: Yellow solid and dot circle (A. and B.) track small-sized (72 nm) and big-sized (213 nm) vesicle respectively. Each shows significantly different kinetic that a small vesicle is ruptured completely whereas a big vesicle remains still with a long lag time. Right: Vesicle size was calculated based on the fluorescence intensity of each vesicle to study the relationship between vesicle size and the rupture of vesicles. Each data point represents an individual single vesicle.

Of note, the peptide concentrations used in fluorescence microscopy experiments and in other experimental techniques may vary because of mainly three reasons: (i) the

model membrane platform design, (ii) the measurement technique's sensitivity, and (iii) the flow conditions among other considerations. For example, the tBLM platform experiments were conducted in non-flow condition without peptide replenishment. By contrast, the tethered vesicle platform experiments were conducted in a microfluidic chamber in a flow-through configuration as controlled by peristaltic pump, whereby fresh peptide was added continually. Therefore, the total amount of peptide that is introduced into the microfluidic chamber during the experiment can be roughly calculated by considering the flow rate, time, and microfluidic chamber volume as well as the bulk peptide concentration. For instance, when 100 nM bulk peptide concentration is injected into the microfluidic chamber for 20 min, it will correspond to roughly 10 μ M total peptide concentration that is introduced into the chamber (100 nM (bulk peptide concentration) * 100 μ L/min (flow rate) * 20 min (measurement time) / 200 μ L (measurement chamber volume)), which is in line with the peptide concentrations used in the no-flow tBLM platform experiments.

Also, the flow rate in this thesis and other studies was mostly set to 100 μ L/min as carefully controlled by peristaltic pump. When originally developing the measurement platform, we did test different flow rates and they influence the timescale of the membrane-peptide interactions. This effect arises from changes in the diffusion flux of peptide molecules in the bulk solution. Thus, we have defined the flow rate in our measurement setup to be 100 μ L/min (as controlled by a peristaltic pump) in order to focus on testing other parameters such as bulk peptide concentration.

3.4.5 Electrochemical Impedance Spectroscopy (EIS)

Within the physiological system, cellular membranes play a role of physical barriers that protect the cell from surrounding environment and regulate permeable ions or molecules. There are various model lipid membrane systems that mimic this electrophysiological properties of biological cellular membranes such as giant unilamellar vesicles (GUVs),⁴⁵ supported lipid bilayers (SLBs),^{46, 47} free-standing lipid bilayers,⁴⁸ and tethered bilayer lipid membranes (tBLMs).²⁰ An engineered tBLM is two-dimensional lipid bilayer structure that are supported by chemical tethering to underlying solid substrate for physical stability. A wide range of surface-sensitive analytical tools including quartz crystal

microbalance with dissipation (QCM-D),^{23, 26, 49} or electrochemical impedance spectroscopy (EIS)²³⁻²⁵ can be used to evaluate the molecular assembly of the tBLMs. Electrochemical impedance spectroscopy (EIS) is a technique that has been utilized by many researchers to characterize electrochemical systems involving corrosion process,^{50, 51} fuel cells⁵² or batteries,⁵³ adsorption/interfacial behaviors,^{54, 55} and more recently, biological applications^{56, 57} as it allows one to monitor the impedance over a range of frequencies in relatively nondestructive way. In biological systems, numerous chemicals dissolved in water (electrolytes) assist the communication among cells in the flow of electrical signals, which can be measured by EIS. By the same principle, the membrane permeability of tBLM caused by membrane-active compounds can be assessed using EIS by measuring the transmembrane voltages across the tethered membrane.

As the membrane is an electrical insulator, the cell membranes or tBLMs are considered as a combination of resistor and capacitor. The EIS setup measures the electrical conductance (G_m) across the tBLM, which is the inverse of its electrical resistance (R_m) and expressed as $G_m = I/V$, where I and V are the current and voltage, respectively. As the voltage is applied across the cell membrane from the counter electrode to tethering electrode (Figure 3.13), a conductance across the tBLM (G_m) will discharge the voltage across the membrane and a partial (~10%) of conductance will be measured as to zero while charging the tethering electrode capacitors. In other words, if there is a pore-forming agent introduced, the measured G_m will increase as more current will pass through the membrane. The setup also measures the capacitance (C_m) of the tBLM, which means the ability of a membrane to store charged ions. The capacitance is expressed as $C_m = q/V$, where q and V are the charge and voltage, respectively, whereby the charge is separated by applying a voltage across the membrane. When membrane leakage occurs, the G_m and C_m values both increase – larger increases indicate greater membrane leakage – and a well-sealed tBLM is important for baseline stabilization.

Figure 3.13 The equivalent circuit for modelling AC electrical impedance data. In this circuit, G_m represents the conduction of the membrane, which is primarily determined by membrane pore defects, C_m is the membrane capacitance.

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Chapter 4* Quantitative Accounting in Single Lipid Vesicle Measurements

Highly parallel measurements on single, tethered lipid vesicles enable real-time monitoring of dynamic membrane interactions of relevance to medical, pharmaceutical, and biotechnological applications. Monitoring the time-dependent release of entrapped fluorescent dyes is a popular measurement approach, although it is often challenging to accurately extract quantitative biochemical parameters. Key issues include dye leakage and imaging-related photobleaching, and corrective measures are needed. Herein, an extended analytical framework to collect and interpret time-lapsed fluorescence microscopy imaging data was presented with its utility for tracking membrane-peptide interactions. This approach is focused on improving platform design and data analysis. First, suitable membrane compositions to minimize dye leakage were identified. Second, a data normalization procedure was developed to correct for experimental artifacts, namely dye leakage and photobleaching, and hence improve measurement accuracy. This analytical procedure was applied to experimentally determine the rate of peptide-induced pore formation in single lipid vesicles, and there was up to a nearly three-fold decrease in the measured rate, as compared to uncorrected data.

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4.1 Introduction

The rational design of nanoscopic soft matter assemblies at solid-liquid interfaces offers excellent potential for developing nanoarchitectonic-based measurement platforms for a wide range of biosensing and biotechnology applications.¹⁻⁴ Towards this goal, surface-based model membranes are useful experimental platforms for characterizing dynamic membrane interactions involving biomacromolecules such as interfacial enzymes and amphipathic peptides.⁵⁻⁶ There are many different types of model membrane platforms, including supported lipid bilayers,⁷ tethered lipid bilayers,⁸ and adsorbed/tethered vesicles,⁹ that mimic key features of biological membranes. Depending on the application, different model membranes have particular sensing merits and adsorbed/tethered lipid vesicles have provided a versatile model system to interrogate the mechanism of action of membrane-active compounds.¹⁰⁻¹²

In some cases, lipid vesicles adsorb via physisorption onto a solid support and form a close-packed layer.¹³ Membrane-active compounds are then added to the adsorbed vesicle layer and numerous surface-sensitive techniques can be utilized to study interaction processes, including acoustic¹⁴ and optical¹⁵ sensors. These approaches focus on measuring time-resolved changes in adlayer properties such as film mass, leading to insights into membrane curvature,¹⁶ lipid composition,¹⁷ and membrane swelling.¹⁸ However, data interpretation is oftentimes challenging because the measurements are conducted on the ensemble-level and influenced by vesicle-substrate and vesicle-vesicle interactions.¹⁹ Hence, there is strong interest in developing surface-based lipid vesicle platforms that enable direct investigation of biomacromolecular interactions at the single-vesicle level.²⁰⁻²²

Towards this goal, the tethering of sub-100 nm lipid vesicles offers excellent potential to prepare surface-based lipid vesicle platforms. The surface is oftentimes functionalized with a passivating layer such that nonspecific vesicle adsorption is nullified while specific tethering of lipid vesicles is aided by high-affinity noncovalent interactions such as biotin-streptavidin,²³ nucleic acid hybridization,²⁴ and alkyl chain attachment.²⁵ The platforms can be tracked using fluorescence microscopy techniques, including confocal,²⁶⁻²⁷ total internal reflection²⁸⁻²⁹ and epifluorescence³⁰ modes, or alternatively

label-free methods such as interferometric³¹ and evanescent³² light-scattering microscopy techniques. In such embodiments, individual vesicles are detected on the basis of fluorescent labels or light scattering properties.³³⁻³⁴ Importantly, time-lapsed imaging capabilities enable highly parallel measurements across thousands of individual lipid vesicles in a single experiment.³⁵

Since dynamic membrane interactions often involve steps such as membrane permeabilization and membrane lysis, there has been interest in developing multi-probe systems whereby individual measurement responses are sensitive to different aspects of interaction processes. For example, a two-fluorophore approach for single-vesicle measurements has been described^{28, 36} that incorporates water-soluble calcein in the vesicle interior and a rhodamine-labeled phospholipid in the vesicle's lipid bilayer. In general, a key premise is that the fluorescence signals are stable in the absence of an added membrane-active compound. However, numerous experimental factors can affect the fluorescence signals, including photobleaching and dye leakage.³⁷⁻³⁸ As a result, there is an outstanding need to improve measurement accuracy by improving platform design along with establishing normalization procedures to correct for experimental artifacts.

Herein, an extended analytical framework to collect and interpret time-lapsed fluorescence microscopy imaging data is presented with its applicability for accurately measuring the rate of peptide-induced pore formation in single lipid vesicles. Systematic experiments identified that dye leakage and imaging-related photobleaching cause experimental artifacts, leading us to employ more stable lipid compositions and develop a data normalization procedure to correct for experimental artifacts.

4.2 Experimental Methods

4.2.1 Reagents

Lipids, including 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG(2000)biotin), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)

(Rh-PE), along with cholesterol (ovine wool) were obtained from Avanti Polar Lipids (Alabaster, AL, USA), and received in chloroform stock solutions. An amphipathic, α -helical (AH) peptide (SGSWLRDVWDWICTVLTDFTWLQSKL-NH₂) was synthesized by Anaspec Corporation (Fremont, CA, USA) and supplied in lyophilized form. A stock solution was prepared by dissolving AH peptide in deionized water, and the molar concentration of peptide was determined by absorbance measurements at 280 nm wavelength. All dilution steps were performed using 10 mM Tris [pH 7.5] buffer solution with 150 mM NaCl (experimental buffer). All solutions were prepared using high-purity, Milli-Q-treated water (MilliporeSigma, Burlington, MA, USA).

4.2.2 Vesicle Preparation

Small unilamellar vesicles (SUVs) were prepared by the extrusion method, as previously described.³⁹ The phospholipid/sterol mixture (DOPC alone or with DOPS or cholesterol) included 0.7 mol % of Rh-PE (maximum excitation and emission wavelengths of 560 and 583 nm, respectively) and 0.1 mol % DSPE-PEG(2000)biotin, and all components were dissolved and mixed in chloroform, and then dried under a stream of nitrogen air. Afterwards, the dried lipid film was incubated overnight under vacuum to remove residual chloroform. Multilamellar vesicles were then generated by hydrating and vortexing the lipid sample in a 10 mM Tris [pH 7.5] buffer solution with 150 mM NaCl at a mass concentration of 2 mg/mL. In the buffer, 14.3 mM calcein dye (maximum excitation and emission properties of 494 and 517 nm, respectively, which is well-suited for tracking applications; see Ref. ⁴⁰) was included to facilitate dye encapsulation within the lipid vesicles. After hydrating the lipid samples, the vesicles were subjected to seven cycles of freeze-thaw treatment to increase encapsulation efficiency and unilamellarity. Then, the lipid suspension was repeatedly passed through a 100-nm diameter polycarbonate filter for a total of 11 times by using a MiniExtruder (Avanti Polar Lipids). The as-prepared vesicle suspensions were diluted right before experiment to a mass concentration of 0.2 mg/mL, and non-encapsulated calcein was removed by a Sephadex G-25 gel filtration column (GE Healthcare Life Sciences, Pittsburgh, PA, USA).²⁸

4.2.3 Tethered Vesicle Platform

Glass coverslips (ibidi GmbH, Martinsried, Germany) were cleaned and treated with oxygen plasma (model no. PDC-002, Harrick Plasma, Ithaca, NY, USA) for 30 sec, before being assembled in a microfluidic chamber (sticky slide VI 0.4, ibidi GmbH). The coverslips were then coated with a mixture of 50/50 wt% poly(L-lysine)-grafted poly(ethylene glycol) (PLL-g-PEG) and poly(L-lysine)-grafted poly(ethylene glycol)-biotin (PLL-g-PEG-biotin) (SuSoS AG, Dübendorf, Switzerland) at a bulk mass concentration of 41.5 $\mu\text{g/mL}$. The incubation time was 30 min followed by rinsing in equivalent buffer solution. Then, the surface was incubated with 10 $\mu\text{g/mL}$ neutravidin for 5 min, followed by buffer washing. Biotinylated vesicles were next immobilized on the functionalized surface by biotin-neutravidin coupling at a bulk mass concentration of 0.025 $\mu\text{g/mL}$. All materials were introduced at a flow rate of 100 $\mu\text{L/min}$ by using a peristaltic pump (Ismatec).

4.2.4 Epifluorescence Microscopy

Imaging experiments were conducted using a Nikon Eclipse Ti-E inverted microscope with a 60 $\times$ oil-immersion objective (NA 1.49). The excitation source was a mercury-fiber illuminator C-HGFIE Intensilight (Nikon, Tokyo, Japan), and the light was passed through an alternating dichroic filter block (Ex 480/40, Em 535/50) or (Ex 545/30, Em 605/70) for imaging in the FITC and TRITC channels, respectively. An Andor iXon3 897 EMCCD camera was used to obtain the images at the rate of 1 frame per 0.4, 1 or 15 min in 4, 8 or 12 neighboring spots, respectively, to minimize thermal drift and loss of focus. The experimental substrate was enclosed within a microfluidic chamber, and liquid sample was introduced at a flow rate of 100 $\mu\text{L/min}$, as controlled by a peristaltic pump (model no. ISM833C, Ismatec, Wertheim, Germany).

4.2.5 Image & Statistical Analysis

The single vesicle image analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD) and an in-house software program written in Python

code, as previously described.^{28, 41} The process is divided into three steps: image adjustment; extraction of fluorescence intensity for individual vesicles; and exponential decay fit to acquire histograms. Image adjustment comprises background removal and image alignment, which is achieved by applying least-squares fits to remove background noise and minimize residual errors across the entire time series. Then, the fluorescence intensity of individual vesicles was extracted by labeling all vesicles that were above a defined threshold value. As described in Ref. ³⁰, defining the threshold is an important step for analyzing the correlation between vesicle size and fluorescence intensity. As a result, the time-resolved data reporting the change in an individual vesicle's fluorescence intensity was exported and fit using a nonlinear (exponential decay) function to obtain the onset of pore formation (as indicated by the calcein signal) or rupture time (as indicated by the rhodamine signal) during the vesicle rupture process.

To compare results between adjacent test groups, the two-tailed t-test was performed in order to calculate p-values ($^*P < 0.05$, $^{***}P < 0.001$). In the box plots, the boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, a circle symbol within the box marks the mean, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers below and above the box indicate the 5th and 95th percentiles, respectively. The number of vesicles analyzed per sample was typically >500 and the standard deviation (s.d.) was defined as $IQR/1.35$, where IQR is the interquartile range. For histogram plots, the mean $\pm$ s.d. was determined by Gaussian fitting and s.d. is defined as $FWHM/2.355$, where FWHM is the full-width-at-half-maximum.

4.3 Results and Discussion

4.3.1 Quantification of Photobleaching Effect

As part of the platform, fluorescently labeled vesicles were tethered to a thin layer of PLL-g-PEG-biotin coating by biotin-neutravidin-biotin coupling (**Fig. 4.1A**).⁴² Vesicles were immobilized at low surface coverage, which enables high spatial discrimination between individual vesicles for monitoring purposes (**Fig. 4.1B**). Up to ~300 vesicles are observed per field of view and highly parallel measurements are attainable by scanning

multiple fields of view in coordinated fashion (**Fig. 4.1C**). The tethered vesicles contained two different fluorescent labels for probing membrane interaction processes. Water-soluble calcein was included in the vesicle interior and a decrease in the calcein signal indicated membrane permeabilization (pore formation),⁴³ while rhodamine-labeled phospholipids were included in the lipid bilayer and a decrease in the rhodamine signal signified membrane lysis.⁴⁴

Figure 4.1 Schematic illustration of two-probe, highly parallel measurement platform for single vesicle analysis. (A) Molecular structures of the lipid and fluorophore components that are used for

constructing the tethered vesicle platform. (B) Top: Schematic illustration of tethered vesicle array for monitoring changes in membrane permeability (water-soluble calcein dye, green; encapsulated within lipid vesicles) and membrane rupture (rhodamine dye-labeled phospholipid, red; in a vesicle's lipid bilayer). Bottom: Time-lapsed imaging is performed, and changes in fluorescence properties of individual, sub-100 nm vesicles can be analyzed. Scale bars are 15 μm . (C) Representative full-view ($136.53 \mu\text{m} \times 136.53 \mu\text{m}$) microscopy images of tethered lipid vesicles. Top: encapsulated water-soluble calcein dye (green) markers. Bottom: Rhodamine dye-labeled phospholipids (red) markers in different excitation/emission channels. Scale bars are 20 μm .

Photobleaching involves the photochemical destruction of fluorophores, and is a cumulative effect that arises from repeated excitation-emission cycles and depends on numerous factors, including dye properties along with exposure frequency, exposure time and excitation energy (light intensity).⁴⁵ In experiments, the frequency of light exposure, which is defined as the imaging (time) interval, is an important parameter to optimize. Therefore, the effects of different imaging time intervals on the fluorescence properties of single vesicles were quantified. During imaging, blue (494 nm) and green (560 nm) fluorescent light were used to excite calcein and rhodamine dyes for 20 and 100 ms, respectively, followed by detection of green or red emitted light at the longer wavelengths of 517 nm (calcein) and 583 nm (rhodamine), respectively (**Fig. 4.2A**). First, the stability of the two fluorescence signals associated with individual vesicles under ambient conditions (buffer flow) for 60 min was checked. Image collection was conducted every 0.4, 1, or 15 mins. The 3D contour plots of the fluorescence intensity profile for a representative vesicle show how the calcein signal typically decreased over time, while the rhodamine signal remained largely stable (**Fig. 4.2B**). In addition, excitation with shorter-wavelength light (*i.e.*, blue light as compared to green light) typically causes greater photobleaching effects as well.⁴⁶

Representative changes in the calcein signal for individual vesicles as a function of imaging interval (0.4, 1, and 15 min) are presented in **Fig. 4.2C**. With increasing frequency of light exposure (shorter imaging interval), the intensity of the calcein signal decreased more quickly and to a greater extent. For each tested imaging interval, >1000 vesicles were monitored in parallel and the calcein intensity for each individual vesicle at the end of the measurement period (at 60 min) was recorded (**Fig. 4.2D**). For the 0.4 and 1-min imaging

intervals, the mean calcein signal dropped to 0.15 ± 0.08 and 0.16 ± 0.08 , respectively. On the other hand, for the 15-min interval, the mean calcein signal was around 0.50 ± 0.20 , indicating that less photobleaching occurred than with the more frequent imaging intervals. Likewise, the dependence on the imaging interval supports that the drop in calcein signal is related to the photobleaching effect.⁴⁷ By contrast, the rhodamine signal for individual vesicles showed minimal changes across the 60-min measurement period for all tested imaging intervals, as presented in **Fig. 4.2E**. The mean rhodamine signal for 0.4, 1, 15 min was 0.94 ± 0.04 , 0.94 ± 0.04 , and 0.95 ± 0.04 respectively (**Fig. 4.2F**). As such, the data indicate that the calcein signal is sensitive to photobleaching effects while the rhodamine signal is less sensitive, which agrees well with the relatively high photostability of rhodamine-based dyes.⁴⁸

Figure 4.2 Time-dependent changes in calcein and rhodamine signals under different imaging interval conditions. (A) Schematic illustration of time-dependent changes in the two measurement signals (B) 3D fluorescence intensity profiles show time-resolved changes in calcein and rhodamine signals. Representative data are shown for a 1-min imaging interval. (C) Time-lapsed changes in the fluorescence intensity of the calcein signal for individual vesicles for different imaging intervals. (D) Statistical comparison of the final intensity of the calcein signal for individual DOPC lipid vesicles at the end of the 60-min measurement period. (E,F) Equivalent data for the rhodamine signal as shown in panels (C,D). *** $P < 0.001$ compared to other groups by two-tailed t-test.

4.3.2 Optimizing Membrane Composition

The effects of membrane composition on the calcein signal response were further investigated. Indeed, calcein can translocate across lipid bilayers without membrane disruption,⁴⁹ especially when lipid vesicles are in the immobilized state.⁵⁰ Therefore, the standard zwitterionic lipid composition was modified so that it is more biologically relevant while also improving the stability of the measurement response, *e.g.*, minimizing the nonspecific leakage of calcein molecules.

4.3.2.1 Effect of Incorporating Anionic Lipids

In order to minimize calcein leakage, incorporation of negatively charged DOPS lipids would be an effective choice because calcein is an acidic molecule (net charge of -4 at pH 7.5; Ref. ⁵¹). Therefore, the effects of incorporating 0–10 mol % DOPS lipid into lipid vesicles on calcein dye leakage were tested. In all cases, the average vesicle diameter was around 115 nm and the lipid compositions were in the fluid-phase state (**Fig. 4.3A**).⁵²

Representative kinetic curves of individual vesicles are presented for the calcein signal response as a function of DOPS lipid fraction (**Fig. 4.3B**). Up to 7.5 mol % DOPS, the rate of decrease in the calcein signal became smaller with increasing DOPS fraction. The trend supports that DOPS lipid incorporation inhibited dye leakage due to electrostatic repulsion between calcein molecules and the lipid bilayer. Interestingly, however, 7.5 mol % DOPS was more effective than 10 mol % DOPS at preventing dye leakage. This observation is likely due to higher DOPS fractions increasing the molecular surface area per lipid headgroup as the result of lateral deformations in the mixed lipid bilayer.⁵³⁻⁵⁴ Hence, there is a balance between inhibiting calcein leakage by promoting electrostatic repulsion between calcein molecules and the lipid bilayer and maintaining structural packing within the lipid bilayer.

Figure 4.3 Influence of DOPS phospholipid incorporation on calcein dye release. (A) Size distribution of vesicles determined by dynamic light scattering (DLS). The intensity-weighted DLS data shows the size distribution (reported as hydrodynamic diameter) for vesicle populations with different DOPS lipid fractions in the membrane composition. The control composition was 100 mol % DOPC. (B) Time-lapsed changes in the fluorescence intensity of the calcein signal for individual vesicles as a function of DOPS lipid fraction, with a 1-min imaging interval. (C) Quantification of the normalized fluorescence intensity of individual DOPC/DOPS lipid vesicles after 60 min. Each data point corresponds to a single vesicle. * $P < 0.05$, *** $P < 0.001$ compared to other groups by two-tailed t-test. (D) Change in fluorescence intensity for individual vesicles after 60-min measurement period as a function of vesicle diameter. The normalized calcein signal intensity, with 0-min baseline, is reported after a 60-min measurement period and plotted as a function of vesicle diameter.

Quantitatively, the incorporation of 2.5 and 5 mol % DOPS lipid led to significant increases in the final fluorescence intensity, with values of 0.34 ± 0.06 and 0.35 ± 0.12 , respectively (**Fig. 3B**). Notably, for the 7.5 mol % DOPS, the final fluorescence intensity decreased to only 0.56 ± 0.14 , while the final value was 0.37 ± 0.09 for the 10 mol % DOPS case. Altogether, the results support that incorporation of DOPS lipid into tethered vesicles can mitigate nonspecific release of calcein dye, and a specific fraction (7.5 mol % DOPS) improved stability of the calcein signal by over 3-fold. The lipid composition was fixed at a 7.5 mol % DOPS fraction for further testing.

4.3.2.2 Effect of Incorporating Cholesterol

Next, the effect of incorporating cholesterol into DOPC/DOPS lipid vesicles was investigated. Cholesterol is a small amphipathic molecule that intercalates within lipid bilayers and regulates membrane fluidity and rigidity, thereby decreasing membrane permeability.⁵⁵⁻⁵⁶ Within the test range of cholesterol fractions (0–7.5 mol %), the average vesicle diameters were in the range of 115–120 nm with a slight increase in diameter depending on the cholesterol fraction (**Fig. 4.4A**),⁵⁵ and the cholesterol-containing membranes can have a coexistence of liquid-disordered and liquid-ordered phases.⁵⁷⁻⁵⁸

Representative kinetic curves of individual vesicles are presented for the calcein signal response as a function of cholesterol fraction (**Fig. 4.4B**). Even small amounts of cholesterol mitigated the nonspecific release of calcein dye, likely due to reinforcing packing between the unsaturated hydrophobic chains of DOPC and DOPS phospholipids within the bilayer.⁵⁹⁻⁶⁰ At higher cholesterol fractions (5 and 7.5 mol %), there was a similar degree of protection against nonspecific calcein dye release. The results are further corroborated by statistical analysis, indicating that, for 2.5 mol % cholesterol, the mean fluorescence intensity after 60 min was 0.67 ± 0.10 , which is significantly higher than the control case without cholesterol (**Fig. 4.4C**). Likewise, the mean fluorescence intensity values for 5 and 7.5 mol % cholesterol were 0.71 ± 0.06 and 0.71 ± 0.09 , respectively.

Figure 4.4 Influence of cholesterol incorporation on calcein dye release. (A) The DLS data shows the size distribution for vesicle populations with different cholesterol fractions in the membrane composition. The control composition was 7.5 mol % DOPS. (B) Time-lapsed changes in the fluorescence intensity of the calcein signal for individual vesicles for different cholesterol fractions, with a 1-min imaging interval. The DOPS lipid fraction was 7.5 mol %. (C) Quantification of the normalized fluorescence intensity of individual DOPC/DOPS/cholesterol lipid vesicles after 60 min. Each data point corresponds to a single vesicle. * $P < 0.05$, *** $P < 0.001$ compared to other groups by two-tailed t-test. (D) Change in fluorescence intensity for individual vesicles after 60-min measurement period as a function of vesicle diameter. The normalized calcein signal intensity, with 0-min baseline, is reported after a 60-min measurement period and plotted as a function of vesicle diameter.

Collectively, the results indicate that optimization of the lipid composition, namely by incorporating specific amounts of DOPS lipid and cholesterol, can reduce the extent of nonspecific calcein dye release while maintaining stable rhodamine signals. Of note, similar performance levels were achieved independently of vesicle size, indicating that membrane optimization is a generic approach for stabilizing tethered vesicles (**Figs. 4.4D** and **4.5D**).

4.3.3 Quantitative Evaluation of a Membrane-Active Peptide

Membrane permeabilization and pore formation – processes which are sensitively detected by the release of entrapped calcein dye molecules – are critical steps involved in the mechanism of action of numerous membrane-active compounds such as amphipathic peptides.⁶¹ Artifact-related decreases in the calcein signal can lead to an overestimation of a membrane-active compound's permeabilizing activity. As such, correcting for artifact-related effects can improve measurement accuracy, motivating us to quantitatively evaluate the membrane-disruptive behavior of an amphipathic, α -helical (AH) peptide as a model system.⁶²

The tested AH peptide is known to form pores in small vesicles (<160 nm diameter) leading to vesicle rupture once a critical density of pores is formed in the lipid bilayer (**Fig. 4.5A**).⁶³⁻⁶⁴ In single-vesicle measurements, AH peptide-induced pore formation and membrane lysis can be tracked by monitoring time-resolved decreases in the calcein and rhodamine signals, respectively (**Fig. 4.5B**). The effects of bulk AH peptide concentration (10–1000 nM) on the time scale of peptide-induced pore formation and corresponding membrane lysis in DOPC lipid vesicles were investigated. This range of peptide concentrations was selected because it is sufficiently high to induce pore-induced vesicle rupture while the resulting interaction process occurs on a suitable timescale to track the corresponding interaction kinetics.^{28, 65}

Figure 4.5 Single-vesicle tracking of AH peptide-mediated pore formation and vesicle rupture. (A) Schematic illustration of AH peptide-induced pore formation and vesicle rupture. (B) 3D fluorescence intensity profiles show time-resolved changes in calcein and rhodamine signals caused by AH peptide. The peptide concentration was 100 nM. Time-lapsed changes in the fluorescence intensity of the (C) calcein and (D) rhodamine signals for individual vesicles that were treated with different AH peptide concentrations, with a 0.4-min imaging interval. Histogram profiles of AH

peptide-mediated pore formation and membrane lysis as a function of bulk peptide concentration. (E) Histograms of drop point in calcein signal for different bulk peptide concentrations. Each binned data point corresponds to an individual vesicle. (F) Histograms of the vesicle rupture (lag) time for different bulk peptide concentrations. Each binned data point corresponds to an individual vesicle. The experiments were conducted using 100 nM AH peptide and data depict mean $\pm$ s.d.

For all tested peptide concentrations, there was a two-step decrease in the calcein signal, namely a nearly linear, gradual decrease followed by a more rapid drop (**Fig. 4.5C**). The initial stage coincides with the onset of pore formation in a lipid vesicle and there is hindered diffusion of dye molecules through one or a few pores, while a more rapid drop began after a critical density of pores was formed in the vesicle and more extensive dye release can thus occur until no dye remains inside the vesicle.^{28, 64} With increasing peptide concentration from 10 nM to 1000 nM, the timescale of calcein release decreased from 7.4 ± 1.2 min to 1.9 ± 0.3 min, as determined by Gaussian curve-fitting analysis of the individual single-vesicle data points plotted in a histogram format (**Figs. 4.5E**). As further demonstrated by tracking the rhodamine signal, 100 nM and higher concentrations of AH peptide led to eventual vesicle rupture whereas 10 nM AH peptide was ineffective (**Figs. 4.5D and F**). Therefore, 100 nM AH peptide was selected for further experiments because this bulk peptide concentration is sufficiently high to cause pore formation-induced vesicle rupture while the time scale is relatively long and hence quantitative evaluation is encumbered by potential measurement artifacts.

To improve measurement accuracy, a procedure was devised to correct for membrane translocation and photobleaching of calcein molecules (**Fig. 4.6A**). Measurement data were collected by tracking the time-resolved changes in calcein signal when 100 nM AH peptide was added to DOPC, DOPC/DOPS, and DOPC/DOPS/cholesterol vesicles (**Fig. 4.6B**, reported as raw data). Under equivalent imaging conditions, the time-resolved change in calcein signal was measured for each membrane composition in the absence of AH peptide and adjusted the experimental data for each membrane composition. Also, there was no significant difference in the kinetic data for similar-size vesicles when subtracting the mean values of the time-resolved change in the calcein signal or when subtracting the time-resolved change in the calcein signal

corresponding to each individual vesicle. The corrected AH peptide interaction kinetics (reported as corrected data) indicate that the native measurement response overestimates the rate of change in the initial calcein drop, and the effect is most prominent for DOPC lipid vesicles. The overestimation in the calcein drop is still significant but to a lesser extent for DOPC/DOPS and DOPC/DOPS/cholesterol vesicles.

Figure 4.6 Corrective measures for single-vesicle analysis of AH peptide-mediated pore formation. (A) Data normalization procedure to correct raw measurement data by removing the effects of dye photobleaching and nonspecific leakage. (B) Time-lapsed changes in the fluorescence intensity of the calcein signal for individual vesicles with different membrane compositions, with a 0.4-min

imaging interval. Raw and corrected profiles refer to measurement data before and after normalization procedure is applied. The peptide concentration was 100 nM. (C) Quantification of the initial rate of change in the calcein signal, without and with correction procedure, for individual vesicles with different membrane compositions. Each data point corresponds to a single vesicle.

*** $P < 0.001$ for the corrected data set compared to the raw data set by two-tailed t-test.

To quantify the magnitude of the corrective effect for each tested membrane composition, the initial rate of change (slope) in the calcein signal for the raw and corrected kinetic profiles of each individual vesicle was determined and compared (Fig. 4.6C). The measured slopes provide an indication of the rate of pore formation and hence the degree of membrane-disruptive activity exhibited by AH peptide. For DOPC lipid vesicles, the native measurement response overestimated the rate of pore formation by a 148% relative error (0.14 ± 0.052 vs. $0.058 \pm 0.051 \text{ min}^{-1}$), whereas the overestimate had a 58% relative error (0.068 ± 0.034 vs. $0.043 \pm 0.030 \text{ min}^{-1}$) in the case of DOPC/DOPS/cholesterol vesicles. Hence, the results indicate that there is up to a three-fold difference in the rate of pore formation depending on the membrane composition and the corrective procedure improves measurement accuracy.

The corrective procedure was also applied to the rhodamine data, although no significant change in the measurement responses was observed (Figure 4.7). As mentioned above, this finding is consistent with greater photostability of the rhodamine dye and its phospholipid anchoring ensures that rhodamine molecules remain bound within the lipid bilayer architecture. Taken together, the results demonstrate how the corrective procedure can improve measurement accuracy in cases where the dye probe is susceptible to membrane translocation/leakage and photobleaching effects. In future work, it will be interesting to further investigate how these corrective strategies can be applied to improve measurement accuracy for characterizing various types of biomacromolecular interaction processes, especially in cases where membranes exhibit raft-like compositions among other possibilities.⁶⁶

Figure 4.7 Corrective measures for single-vesicle analysis of AH peptide-mediated membrane lysis. (A) Time-lapsed changes in the fluorescence intensity of the rhodamine signal for individual vesicles with different membrane compositions, with a 0.4-min imaging interval. Raw and corrected profiles refer to measurement data before and after the normalization procedure is applied. The peptide concentration was 100 nM. (B) Quantification of the lag time of the rhodamine signal, without and with correction procedure, for individual vesicles. Each data point corresponds to a single vesicle.

4.4 Conclusions

Our findings demonstrate that experimental artifacts such as dye leakage and imaging-related photobleaching can influence quantitative interpretation of measurement data in single lipid vesicle measurements. By taking into account the physicochemical properties of the tested fluorescent dyes and rationally optimizing the membrane composition, broadly applicable strategies to improve measurement accuracy were identified based on minimizing nonspecific dye leakage and normalizing experimental data

to correct for experimental artifacts. The extended analytical framework was applied to accurately measure the rate of peptide-induced pore formation in individual, sub-100 nm lipid vesicles as a model system. Looking forward, these analytical capabilities will prove useful for single lipid vesicle measurements.

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Chapter 5* Micropatterned Viral Membrane Clusters for Antiviral Drug Evaluation

The function of biological nanoparticles, such as membrane-enveloped viral particles, is often enhanced when the particles form higher-order supramolecular assemblies. While there is intense interest in developing biomimetic platforms that recapitulate these collective properties, existing platforms are limited to mimicking individual virus particles. Here, a micropatterning strategy is presented to print linker molecules selectively onto bioinert surfaces, thereby enabling controlled tethering of biomimetic viral particle clusters across defined geometric patterns. By controlling the linker concentration, it is possible to tune the density of tethered particles within clusters while enhancing the signal intensity of encapsulated fluorescent markers. Time-resolved tracking of pore formation and membrane lysis revealed that an antiviral peptide can disturb clusters of the membrane-enclosed particles akin to the targeting of individual viral particles. This platform is broadly useful for evaluating the performance of membrane-active antiviral drug candidates while the micropatterning strategy can be applied to a wide range of biological nanoparticles and other macromolecular entities.

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5.1 Introduction

The development of highly parallel, surface-based experimental platforms to investigate nanoscopic lipid vesicles and other biological nanoparticles such as exosomes and viruses has opened the door to understanding a wide range of membrane-related chemical reactions,^{1, 2} enzymatic processes,³ and biophysical activities.⁴ One of the most powerful measurement platforms involves the site-specific tethering of well-separated vesicles for real-time monitoring of biomacromolecular interaction processes *via* fluorescence⁵ or scattering^{6, 7} microscopy techniques. These tethering strategies mimic those used for selective molecular capture surfaces using chemically functionalized surfaces.⁸⁻¹² The vesicles are synthetic, spherical lipid bilayer nanoparticles and mimic the architecture of complex biological nanoparticles such as membrane-enveloped viruses while providing a well-controlled platform to investigate dynamic membrane interactions. Importantly, each individual vesicle can be studied in parallel, which overcomes challenges with ensemble averaging and enables insights with high statistical significance.^{13, 14}

Using a two-fluorophore system to simultaneously track membrane permeation and lysis simultaneously, time-lapsed fluorescence microscopy experiments revealed that membrane curvature beyond a certain threshold triggers the formation of pores in individual, enveloped virus-mimicking vesicles by an amphipathic, α -helical (AH) peptide.¹⁵ Following similar experimental approaches, it was later discovered that the membrane curvature sensing exhibited by AH peptide is rather unique compared to other antiviral peptides¹⁶ and peptide engineering could further modulate the degree of membrane curvature sensing.¹⁷ Furthermore, the AH peptide has been shown to exhibit similar membrane-disruptive behavior against zwitterionic, positively charged, and negatively charged vesicles.¹⁸ Importantly, these biophysical findings obtained with virus-mimicking model vesicles were translated into a novel therapeutic strategy called Lipid Envelope Antiviral Disruption (LEAD) whereby an engineered AH peptide was demonstrated to inhibit viral particles *in vivo*.¹⁷

There is intense interest in understanding how biological nanoparticles, such as viruses, form cooperative assemblies with heightened functionalities.¹⁹ One of the most important examples concerns cellular infection by viral particles. Oftentimes, individual

viral particles have relatively low infection efficiencies while clusters of viral particles can display greater infectivity levels.^{20, 21} Interestingly, this cooperative activity has evolved independently across multiple viral families.²² Cooperative assemblies (termed “clusters” here) can consist of aggregates of viral particles alone²³ or enclosed within vesicular bodies.^{24, 25} Other evidence also points to the importance of viral particle clusters, such as the observation that amyloid fibrils can enhance sexual transmission of numerous viruses by inducing aggregation of viral particles.²⁶ Indeed, it has long been recognized that it is difficult to neutralize clusters of viral particles²⁷ and the cluster can also exhibit high environmental stability.²⁸ Altogether, these findings underscore the importance of developing therapeutic strategies aimed at targeting clustered viral particles.

Figure 5.37 Schematic illustration of the experimental strategy. Micropatterned viral membrane clusters mimic the architecture of viral particle clusters, which often display heightened

functionality compared to individual particles. The clusters consist of enveloped virus-mimicking vesicles akin to viral particle aggregates.

To date, however, existing measurement platforms have focused on tethering individual vesicles in non-clustered formats while the nonspecific adsorption of vesicles in close-packed adlayers is limited to ensemble-averaged readouts. The development of a measurement platform that mimics the architectural configuration of clustered viral particles, *i.e.*, collections of nanoscopic, membrane-enclosed objects in grouped arrays, and enables cluster-by-cluster measurement analysis would greatly facilitate mechanistic understanding of how the LEAD strategy can be deployed against viral clusters and other aggregates of biological nanoparticles (**Fig. 5.1**).

5.2 Experimental Methods

5.2.1 Reagents

Lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA), which were received in either chloroform solution or powder form. Lipids included 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG(2000)biotin), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-PE). The Rh-PE lipid is a fluorescently labeled lipid whereby the lissamine rhodamine B sulfonyl probe is covalently conjugated to the phosphoethanolamine headgroup. All reagents were mixed in chloroform to the desired molar ratio as part of the vesicle preparation, as described below. An amphipathic, α -helical (AH) peptide (SGSWLRDVWDWICTVLTDFKTWLQSKL-NH₂) was synthesized by and obtained from Anaspec Corporation (Fremont, CA, USA). The lyophilized peptide was first solubilized in 4% dimethyl sulfoxide (DMSO >99.9%) and then diluted with deionized water to prepare a stock solution (2 mg/ml). Molar concentrations were measured by standard absorbance measurements at 280 nm.²⁹ The buffer used in all experiments was 10 mM Tris buffer with 150 mM NaCl (pH 7.5). Buffers and solutions were prepared with

Milli-Q-treated water (MilliporeSigma, Burlington, MA, USA).

5.2.2 Vesicle Preparation

Small unilamellar vesicles were prepared by the extrusion method, as previously described.³⁰ The lipid composition was DOPC 99.2 mol %, Rh-PE 0.7 mol %, and DSPE-PEG(2000)biotin 0.1 mol %. Briefly, lipids dissolved in chloroform were first dried under a gentle stream of nitrogen air at room temperature, then stored under vacuum overnight to remove residual chloroform. The dried lipids were hydrated in a buffer solution containing 14.3 mM calcein, followed by seven cycles of freeze-thaw treatment to increase the encapsulation efficiency and unilamellarity. Then, the lipid suspension was passed through 50 nm diameter pore, track-etched polycarbonate membranes for a total of 21 times by a MiniExtruder (Avanti Polar Lipids). Vesicle suspensions were diluted immediately before experiment to 0.2 mg/ml, and free calcein was removed by a Sephadex G-25 gel filtration column (GE Healthcare Life Sciences, Pittsburgh, PA, USA). After vesicle preparation, the size distribution of extruded vesicles was obtained by dynamic light scattering with a ZetaPals Particle Size Analyzer (Brookhaven Instruments, Holtsville, NY, USA).

5.2.3 PDMS Stamp Fabrication

A 500 nm SiO₂ film was thermally grown on a Si(100) wafer. Conventional photography was performed to generate a pattern of a 2D array of squares (2 μm in length and 3.5 μm pitch) on the photoresist coated on the SiO₂/Si(100) wafer. After that, the exposed SiO₂ was selectively etched by reactive ion etching (Oxford 80 Plus) using a gas mixture of CHF₃ (25 sccm) and Ar (25 sccm) at 35 mTorr. Next, exposed Si(100) was anisotropically etched in a mixture of 4:1 KOH (30%) and isopropanol at 75°C to generate a 2D array of recessed pyramidal structures. After removing the remaining SiO₂ and photoresists in a 25% HF solution for 5 min, Si masters with recessed pyramidal structures were obtained. Next, Sylgard® 184 elastomer silicone elastomer base and curing agent (in a mass ratio of 10:1) were well mixed, degassed, poured onto the Si masters, and cured at 65°C overnight. After curing, polydimethylsiloxane stamps carrying a 2D array of

pyramidal structures were formed and then it was carefully peeled off from the Si master.

5.2.4 Microcontact Printing (μ CP)

Glass microscope coverslips and polydimethylsiloxane (PDMS) stamps were cleaned by 1 wt% sodium dodecyl sulfate (SDS), deionized water, and ethanol in sequence, followed by 30 seconds of oxygen plasma treatment (Harrick Plasma, Ithaca, NY, USA) to render the surface hydrophilic and remove residual organic contaminants. Then, the glass substrate was coated with poly(L-lysine)-grafted poly(ethylene glycol)-biotin (PLL-g-PEG-biotin 50%, SuSoS AG, Dübendorf, Switzerland, 41.7 μ g/ml diluted in buffer solution). The stamp was immersed with a few drops (15–20 μ l) of stamping solution (ink containing defined neutravidin protein concentrations) for 5 min. Then, the stamp was dried by inert nitrogen gas and placed in contact with the coverslip for a few seconds to transfer neutravidin onto the biotinylated glass surface. Of note, the time interval between drying the ink on the stamp and ink printing was kept as short as possible because the transfer success decreases significantly after 1 min of drying.³¹ Then, a sticky-slide VI^{0.4} (ibidi GmbH, Martinsried, Germany) was attached onto the stamped coverslip to form the microfluidic chamber. Then, each chamber was washed with more than 2 ml of buffer prior to 5 min incubation with 0.025 μ g/ml biotinylated vesicles. As for the control experiments with non-patterned samples, all procedures were the same, except neutravidin was incubated directly in the microfluidic chamber for 20 min.

5.2.5 Epifluorescence Microscopy

Imaging experiments were conducted using a Nikon Eclipse Ti-E inverted microscope with a 60 $\times$ oil-immersion objective (NA 1.49). The excitation source was a mercury-fiber illuminator C-HGFIE Intensilight (Nikon, Tokyo, Japan), and the light was passed through an alternating dichroic filter block (Ex 480/40, Em 535/50) or (Ex 545/30, Em 605/70) for imaging in the FITC and TRITC channels, respectively. An Andor iXon3 897 EMCCD camera was used to obtain the images at the rate of 1 frame per 24 s in 4 neighboring spots, respectively, to minimize thermal drift and loss of focus. The

experimental substrate was enclosed within a microfluidic chamber, and liquid sample was introduced at a flow rate of 100 $\mu\text{L}/\text{min}$, as controlled by a peristaltic pump (model no. ISM833C, Ismatec, Wertheim, Germany).

5.3 Results and Discussion

5.3.1 Design Rationale

5.3.1.1 Conventional Single Vesicle Platform

Conventionally, tethered vesicles contain a small fraction of biotinylated lipid and are attached to biotin moieties on a PLL-g-PEG/PLL-g-PEG-biotin³² functionalized glass surface *via* neutravidin coupling (**Fig. 5.2A**). In this case, a bulk solution of neutravidin is first incubated with the functionalized surface, and neutravidin molecules binds universally across the surface to biotin moieties and then the biotinylated vesicles are added for subsequent coupling in a sandwich configuration. Individual vesicles can be identified by epifluorescence microscopy, whereby water-soluble calcein molecules (green fluorescence) are encapsulated within the liposomal interior and rhodamine-labeled phospholipids (red fluorescence) form part of the liposomal bilayer (**Fig. 5.2B**). Hence, drops in the calcein and rhodamine signals can indicate membrane permeation and lysis, respectively.

Figure 5.38 Design of the viral membrane cluster platform. (A) Schematic illustration of tethering individual vesicles. (B) Fluorescence microscope images of individually tethered vesicles (~100 nm diameter), as indicated by calcein and rhodamine signals. All scale bars are 10 μm .

5.3.1.2 Micropatterned Viral Membrane Clusters

These basic sensing principles to develop a hierarchically assembled cluster platform by employing microcontact printing (μCP).^{31, 33} The strategy is based on fabricating a polydimethylsiloxane (PDMS) stamp with precisely fabricated relief structures that define a printing pattern (**Fig. 5.3A**). The PDMS stamp was rendered hydrophilic by oxygen plasma treatment and coated with a neutravidin “ink” that can be

printed onto target surfaces in patterns defined by the relief features on the stamp. Atomic force microscopy measurements indicate that the PDMS stamp consists of equidistantly spaced, pyramidal tips with heights and widths of 0.9 and 2 μm , respectively, and a separation distance of 1.5 μm between nearest-neighbor edges, as defined lithographically into the master from which the stamp was produced (**Figs. 5.3B** and **C**). These geometrical dimensions are suitable for robust printing, as demonstrated by the controlled printing of fluorescently labeled neutravidin on a bare glass surface (**Fig. 5.3D**).

Figure 5.39 Microcontact printing. (A) Schematic illustration describing microcontact printing (μCP) for location-specific patterning of neutravidin protein molecules. (B) Atomic force microscope (AFM) image of a polydimethylsiloxane (PDMS) stamp with pyramidal stamp pattern. (C) Height profile from AFM scan corresponding to PDMS stamp shown in (B). (D) Fluorescence microscope images of FITC-labeled neutravidin on a bare glass substrate. A scale bar is 10 μm .

Using unlabeled neutravidin (10 $\mu\text{g/ml}$), the μCP strategy was next applied to a PLL-g-PEG/PLL-g-PEG-biotin functionalized glass surface (**Fig. 5.4A**). The functionalized glass surface was treated with the neutravidin-coated PDMS stamp in order to immobilize neutravidin molecules selectively, in the target regions only. Each contacting substrate was well-hydrated to ensure effective transfer of neutravidin molecules from a nonspecific adsorbed state on the PDMS stamp to being captured by biotin moieties on the functionalized glass surface. While μCP methods typically involve physical deposition or pre-functionalization,³⁴ the selective transfer of neutravidin in our case is more elaborate because the protein molecules are being transferred to a nominally bio-inert surface and the specific attachment is mediated by biotin moieties with the latter functionalization occurring after deposition. This distinction is important because our approach enables the subsequent attachment of high densities of fluorescently labeled vesicles in neutravidin-printed regions while other surrounding regions are devoid of vesicles, as confirmed by epifluorescence microscopy (**Fig. 5.4B**). Both the rhodamine and calcein signals indicate that the tethered vesicles faithfully mimic the chemical pattern, pointing to the successful formation of a measurement platform comprising clusters of virus-mimetic vesicles. Compared to the non-patterned platform, the μCP strategy also overcomes the incubation time and reagent concentration requirements of diffusion-limited attachment schemes, thereby enabling efficient transfer with minimal reagents in a small volume.

Figure 5.40 (A) Schematic illustration describing μ CP strategy to form micropatterned viral membrane clusters. (B) Fluorescence microscope images of patterned vesicles on a PLL-g-PEG/PLL-g-PEG-biotin functionalized glass surface, as indicated by calcein and rhodamine signals. All scale bars are 10 μ m.

5.3.2 Effect of Neutravidin Concentration on Tethered Density

Next, the influence of varied amount of bound neutravidin to the formation of virus-mimetic clusters was investigated, as presented in **Figure 5.5**. The experiments were conducted in comparative fashion across the micropatterned cluster and conventional, non-

patterned platforms; ~62-nm diameter vesicles were detected by the calcein and rhodamine signals. The reported neutravidin concentrations correspond to the bulk protein concentration that was used during the appropriate platform fabrication step. In the absence of neutravidin, there was negligible vesicle immobilization on either platform, consistent with the nonfouling characteristics of the PLL-g-PEG/PLL-g-PEG-biotin layer.

With 0.1 $\mu\text{g/ml}$ neutravidin, trace vesicles were immobilized on the non-patterned platform, while some clusters were formed on the micropatterned platform. Notably, the clusters exhibited wide variances in fluorescence intensities and were irregularly distributed across the surface. With 1 $\mu\text{g/ml}$ neutravidin, there were greater densities of immobilized vesicles on the non-patterned platform, although appreciably more vesicles were detected within the same field of view by the rhodamine signal than the calcein signal. This difference might be attributed to the small vesicle size and resulting low amount of encapsulated calcein molecules.³⁵ Likewise, clusters on the micropatterned platform began to assume more uniform characteristics, as indicated by the correlation of the cluster location with the designed chemical pattern. However, there was still high variances in the fluorescence intensities per cluster, especially for the calcein signal.

For the non-patterned platform, similar trends were observed with 10 $\mu\text{g/ml}$ neutravidin and there were high densities of immobilized vesicles, albeit the number of vesicles detected by the rhodamine signal was appreciably higher than the number detected by the calcein signal. In contrast, cluster formation on the patterned platform exhibited high degrees of uniformity, as indicated by cluster locations as well as more similar numbers of clusters being detected by the calcein and rhodamine signals (Figs. 5.6 and 7). The intensity values were also higher than those obtained with lower neutravidin concentrations, indicating that greater densities of vesicles were immobilized per cluster. On the other hand, with 100 $\mu\text{g/ml}$ neutravidin, nearly complete saturation of the surface was observed with the non-patterned platform. While a granular appearance in the fluorescence microscopy images suggests the presence of immobilized vesicles, the high density of immobilized vesicles obfuscates intensity quantification. In marked contrast, the patterned platform had nearly uniform clusters across the entire surface and each cluster could be discerned individually. In general, the fluorescence intensity within the printed spots on the patterned platform was higher than the fluorescence intensity of individual vesicles on the non-

patterned platform. The increased fluorescence intensity is related to a higher surface density of tethered vesicles as the result of the efficient μ CP process.³⁶ The advantages of building spatially encoded internal controls into such measurements were shown in other work.³⁷⁻³⁸

Figure 5.41. Fluorescent microscope images comparing immobilization of tethered vesicles in single-vesicles and clustered vesicle arrangements. The calcein and rhodamine signals are presented for each experimental set. During fabrication, the bulk neutravidin concentration was varied from 0 to 100 $\mu\text{g/mL}$ in order to control the density of tethered vesicles. The number of tethered vesicles or clusters identified by the calcein or rhodamine signals are reported in the upper right image and are based on imaging in ten different fields of view. For the non-patterned platform, it was not

possible to quantify the number of individual vesicles for the 100 $\mu\text{g/ml}$ neutravidin case. All scale bars are 20 μm .

Figure 5.42 Fluorescence intensity quantification of liposome clusters. Particle counting procedure with full image view ($137 \times 137 \mu\text{m}^2$) of (A) control experiment (0 $\mu\text{g/ml}$ neutravidin), (B) 0.1 $\mu\text{g/ml}$, (C) 1 $\mu\text{g/ml}$, (D) 10 $\mu\text{g/ml}$, and (E) 100 $\mu\text{g/ml}$ neutravidin cases. (F) Pixel-by-pixel fluorescence intensity quantification showing the liposome clusters have higher intensities with higher bulk neutravidin concentrations during the fabrication process. Higher fluorescence intensity is related to greater density of tethered liposomes. All scale bars are 20 μm .

Figure 5.43 Quantification of tethered liposomes on non-patterned and patterned platforms. Particle counting results for single liposomes and liposome clusters on the non-pattern and patterned platforms, respectively.

5.3.3 Interaction between AH Peptide and Viral Membrane Clusters

Next, the interaction of 100 nM AH peptide with micropatterned virus-mimetic clusters prepared was investigated using different neutravidin concentrations and hence varying vesicle densities within individual clusters. Peptide-induced pore formation was investigated by tracking changes in the calcein signal. Representative time-lapsed micrographs for the 10 μg/ml neutravidin case are presented in **Figure 5.8A** and the time-dependent drop in the calcein signal per cluster indicates that the AH peptide is capable of causing membrane permeabilization in clustered vesicles. Indeed, AH peptide induced complete release of encapsulated calcein across all tested cluster densities (**Fig. 5.8B**). With increasing neutravidin concentration, the time scale of complete calcein release was slower, supporting that the AH peptide likely takes a longer time to penetrate the entire cluster of vesicles due to hindered diffusion of peptide molecules through the liposomal matrix as compared to through the bulk solution.³⁹⁻⁴⁰ The AH peptide selectively forms pores in small (high-curvature) vesicles,⁴¹⁻⁴² reinforcing that the ~62-nm diameter vesicles maintain their individual architectures within the collective assembly. Of note, the onset of calcein release

was only weakly dependent on the neutravidin concentration, consistent with the outer vesicles being permeabilized more quickly than vesicles within the cluster (**Fig. 5.8C**).

In addition to tracking pore formation, membrane lysis by AH peptide was further interrogated by monitoring the rhodamine signal. Representative time-lapsed micrographs for the 10 $\mu\text{g/ml}$ neutravidin case are presented in **Figure 5.8D**. The progressive decrease in the fluorescence intensity of individual clusters indicates that AH peptide causes complete membrane lysis. Across the range of tested neutravidin concentrations, AH peptide induced drops in the rhodamine signal (**Fig. 5.8E**). Up to 10 $\mu\text{g/ml}$ neutravidin, AH peptide caused complete membrane lysis of the clusters, while incomplete lysis occurred at 100 $\mu\text{g/ml}$ neutravidin-generated patterns. The latter finding supports that AH peptide is effective at disrupting clusters up to a certain vesicle density but the rupture efficiency becomes lower at higher vesicle densities. With increasing neutravidin concentration, the time scale of vesicle rupture also become longer (**Fig. 5.8F**). This trend presumably relates to the greater number of tethered vesicles along with the higher density of vesicles within each individual cluster.

Figure 5.44. Time-resolved tracking of AH peptide interaction with micropatterned viral membrane clusters. (A) Time-lapse fluorescent microscope images show the change in calcein signal when 100 nM AH peptide was added to clusters at $t = 0$ min. During fabrication, the bulk neutravidin concentration was 10 μg/ml. (B) Changes in normalized calcein signal intensity of a representative, individual cluster for platforms fabricated using different neutravidin concentrations with the time-resolved release of the calcein marker. (C) Box plot graph reporting trend in calcein release times. Data correspond to experiments presented in (B). Each dot represents one cluster and mean values are reported from Gaussian fitting. (D-F) Corresponding data are presented for the rhodamine signal in the same experimental series. All scale bars are 20 μm. Control indicates non-patterned platform that was fabricated using a 10 μg/ml neutravidin concentration.

5.4 Conclusions

In summary, virus-mimetic clusters of tethered vesicles can be assembled using microcontact printing. This platform has significant advantages over conventional single-vesicle arrays in terms of material requirements, fabrication speed, and control over platform architecture. Following this approach, one can track the time-resolved interactions between individual clusters and a membrane-disrupting antiviral peptide, revealing how viral particle clusters are susceptible to peptide treatment. This cluster platform is representative of viral particle aggregates, while other types of clusters exist that include a giant vesicle cloaking the aggregated viral particles. Since it is known that the AH peptide can translocate the lipid bilayer of giant unilamellar vesicles,⁴³ it will be interesting to extend these studies to *in vitro* experimental settings in order to determine how the results obtained with this engineering platform correlate with a drug's inhibitory activity against viral particle aggregates and/or giant vesicle-cloaked viral particle assemblies. Such insights complement the insights obtained using conventional single-vesicle arrays and open the door to understanding the biochemical and biophysical interactions involving clusters of biological nanoparticles such as viruses. In future work, these measurement capabilities will also be useful for further exploring how membrane factors such as lipid composition influence the activity profile of antiviral drug candidates.

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Chapter 6* Comparing the Membrane Interaction Profiles of Two Antiviral Peptides: Insights into Structure-Function Relationship

In recent years, certain amphipathic, α -helical peptides have been discovered that inhibit medically important enveloped viruses by disrupting the lipid membrane surrounding individual virus particles. Interestingly, only a small subset of amphipathic, α -helical peptides demonstrates inhibitory activity, and there is broad interest in understanding how the structures of these peptides contribute to functional activity against lipid membranes. To address this question, herein, multiple surface-sensitive measurement techniques were employed along with computational simulations in order to investigate how AH and C5A peptides – two of the most biologically active peptides in this class – interact with model lipid membranes while gaining insight into membrane-induced peptide conformational changes. Taken together, the findings demonstrate that membrane-active antiviral peptides can exhibit distinct membrane interaction profiles that confer different degrees of targeting selectivity and the corresponding structural insights will be useful for peptide engineering applications.

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6.1 Introduction

Membrane-active peptides are important anti-infective agents that target infectious pathogens by destabilizing phospholipid membrane coatings.¹⁻³ The classical description of anti-infective peptides typically focuses on cationic antimicrobial peptides that selectively destabilize membranes enriched in negatively charged lipids (*e.g.*, bacterial cell membranes).⁴⁻⁵ In recent years, there has been increased attention on another exciting class of anti-infective peptides termed antiviral peptides, which can directly impair the lipid membrane surrounding enveloped virus particles.⁶ This antiviral approach has several compelling features, including broad-spectrum inhibitory potential against multiple classes of enveloped viruses and there is a high barrier to the emergence of drug-resistant virus strains.⁷⁻⁸ While the disruption of enveloped viruses by various classes of membrane-active agents (*e.g.*, surfactants,⁹⁻¹⁰ alcohols,¹¹ photosensitizers¹²) has long been known to reduce infectivity *in vitro*, recent evidence points to the *in vivo* and *ex vivo* efficacy of membrane-active antiviral peptides¹³⁻¹⁴ and thus steps closer to realize potential translational opportunities. Together, these inhibitory activities have led to the paradigm concept of Lipid Envelope Antiviral Disruption (LEAD),⁸ whereby an antiviral peptide-induced reduction in the extracellular concentration of infectious virus particles can lead to medically beneficial outcomes.

Within this scope, there is significant interest in understanding the basic design principles of membrane-active antiviral peptides, including how the structural properties of a peptide contribute to functional activity and the physicochemical basis of targeting selectivity.^{13, 15} Since the lipid membrane surrounding enveloped viruses is derived from host cell membranes, the lipid compositions of viral membranes and infected cell membranes can be similar and hence antiviral peptides need to have different mechanisms of targeting selectivity.¹⁶ Unlike the cationic antimicrobial peptides described above, two of the most widely studied, membrane-active antiviral peptides, the 27-mer AH and 18-mer C5A,¹⁷ have net charges of 0 and -2 , respectively. Interestingly, the AH and C5A peptides have partially overlapping amino acid sequences and are both derived from the N-terminus of the hepatitis C virus (HCV) nonstructural protein 5A (NS5A) which plays an important role in HCV genome replication, as illustrated in **Figures 2.2** and **2.4**.

Herein, using multiple surface-sensitive measurement technique and computational simulations, the membrane-interaction profiles of AH and C5A peptides were characterized with model lipid membranes and sought to understand how peptide conformational changes drive these membrane interactions. This experimental approach applied the nanoarchitectonics concept, whereby two different types of supported lipid membrane platforms were selected, lipid vesicles with different degrees of membrane curvature and a tethered lipid bilayer membrane (tBLM), in order to characterize the membrane-peptide interactions using different measurement techniques.¹⁸⁻²¹ To this end, circular dichroism spectroscopy experiments were conducted to determine peptide secondary structure in aqueous buffer and in the presence of lipid membrane environments, leading to insights into coil-to-helix folding and the thermodynamics of membrane-peptide interactions. These results were complemented by single-vesicle assay measurements to characterize the real-time interactions of the two peptides with lipid vesicles along with electrochemical impedance spectroscopy measurements to determine to what extent the peptide interactions cause membrane leakage in the tBLM configuration. Together, these experimental studies build a comprehensive picture of how AH and C5A peptides distinctly interact with lipid membranes, and additional computational simulations further provided insight into how these functional differences might relate to peptide conformational structure.

6.2 Experimental Methods

6.2.1 Reagents

High-purity AH and C5A peptides (96% and 95%, respectively) were synthesized by AnaSpec Corporation (Fremont, CA, USA). The lyophilized peptide was weighed and solubilized in deionized water to prepare a highly concentrated stock concentration (2 mg/ml). The molar concentrations of the two peptides were determined by absorbance measurements at 280 nm wavelength and a Boeco-S220 spectrophotometer (Boeco, Hamburg, Germany) was used for the experiments.²² The experimentally determined molar concentrations of AH and C5A peptides were 554 μ M and 782 μ M, respectively, which indicate that both peptides have high solubility in water. The peptide stock aliquots were

stored at -20°C and diluted in buffer solution before experiment. Lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA), and were supplied as a dispersion in a chloroform solution or in a lyophilized, powder form. Lipids included 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) for all experiments, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG(2000)biotin), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-PE) were used for tethered vesicle experiments. All of the lipid reagents were mixed in chloroform to the desired molar ratio before vesicle preparation as described below. The aqueous buffer was 10 mM Tris [pH 7.5] buffer with 150 mM NaCl unless otherwise noted. All solutions were prepared using Milli-Q-treated water (MilliporeSigma, Burlington, MA, USA).

6.2.2 Circular Dichroism (CD) Spectroscopy

The secondary structure of peptides was characterized by CD spectroscopy experiments using a Chirascan spectropolarimeter (Model 420, AVIV Biomedical Inc., Lakewood, NJ, USA) with a 0.1 cm path length cuvette (Hellma GmbH, Müllheim, Germany). The test peptide concentration was fixed at 100 μM . Mixtures of peptide and lipid were mixed to the appropriate concentration (*e.g.*, P:L ratio of 1:10 corresponded to 100 μM peptide and 1 mM lipid), and then incubated for 30 min before experiment. The aqueous buffer used for all steps was 10 mM Tris [pH 7.5] buffer with 150 mM NaCl. The maximum lipid concentration was set at 2 mM, which is within the optimal range for data collection.²³ Spectral data were collected in triplicate at 25 $^{\circ}\text{C}$, and the wavelength range was 190 to 260 nm with 1 nm bandwidth, a step size of 0.5 nm, and a time constant of 0.1 s. Background scans without peptide were recorded using the same conditions and subtracted accordingly. The final averaged spectra were calculated in mean residue molar ellipticity units ($[\theta] = \theta / 10 \times c \times l$), where θ is the ellipticity, c is the molar concentration of peptide, and l is the path length in centimeter. The presented spectra were smoothed by the Savitzky-Golay method with a polynomial order of two. The fractional helicity (fH) of the peptides was calculated as follows

$$fH = \frac{[\theta]_{222}^{obs} - 3000}{-36000 - 3000}$$

where $[\theta]_{222}^{obs}$ is the molar ellipticity at 222 nm.^{15, 24}

6.2.3 Tethered Vesicle Platform

Glass coverslips (ibidi GmbH, Martinsried, Germany) were cleaned and treated with oxygen plasma (model no. PDC-002, Harrick Plasma, Ithaca, NY, USA) for 30 sec, before being assembled in a microfluidic chamber (sticky slide VI 0.4, ibidi GmbH). The coverslips were then coated with a mixture of 50/50 wt% poly(L-lysine)-grafted poly(ethylene glycol) (PLL-g-PEG) and poly(L-lysine)-grafted poly(ethylene glycol)-biotin (PLL-g-PEG-biotin) (SuSoS AG, Dübendorf, Switzerland) at a polymer mass concentration of 41.5 µg/ml. The incubation time was 30 min followed by rinsing in 10 mM Tris [pH 7.5, 150 mM NaCl] buffer. Then, the surface was incubated with 0.17 µM neutravidin for 5 min, followed by buffer washing. Biotinylated vesicles were next immobilized on the functionalized surface by biotin-neutravidin coupling at a lipid concentration of ~0.03 µM. Image analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD) and an in-house software program written in Python code, as previously described.¹⁵

6.2.4 Vesicle Preparation

For tethered vesicle platform, small unilamellar vesicles (SUVs) were prepared by the extrusion method, as previously described.²⁵ Dissolved lipids (99.2 mol % DOPC, 0.7 mol % of Rh-PE and 0.1 mol % of DSPE-PEG(2000)biotin) in chloroform were first dried under a gentle stream of nitrogen air at room temperature, and were then stored under vacuum conditions overnight to remove any remaining chloroform by evaporation. Multilamellar vesicles were then generated by hydrating and vortexing the dried lipids in aqueous buffer to achieve a bulk lipid concentration of ~2.5 mM. The buffer contained 14.3 mM calcein dye (maximum excitation and emission wavelengths of 494 and 517 nm, respectively), which was thus incorporated into the lipid vesicles. After hydrating the lipid samples, the vesicles were subjected to seven cycles of freeze-thaw treatment to increase

encapsulation efficiency and unilamellarity. Then, the lipid suspension was repeatedly passed through a polycarbonate filter for a total of 11 times by using a MiniExtruder (Avanti Polar Lipids). Depending on the diameter of pores within the filter, the average diameter of extruded vesicles was 126 and 313 nm for 100-nm and 400-nm filters, respectively, as determined by dynamic light scattering. The as-prepared vesicle suspensions were diluted before experiment to a lipid mass concentration of ~0.25 mM, and non-encapsulated calcein was removed by a Sephadex G-25 gel filtration column (GE Healthcare Life Sciences, Pittsburgh, PA, USA).²⁶

6.2.5 Epifluorescence Microscopy

Imaging experiments were conducted using a Nikon Eclipse Ti-E inverted microscope with a 60× oil-immersion objective (NA 1.49). The excitation source was a mercury-fiber illuminator C-HGFIE Intensilight (Nikon, Tokyo, Japan), and the light was passed through an alternating dichroic filter block (Ex 480/40, Em 535/50) or (Ex 545/30, Em 605/70) for imaging in the FITC and TRITC channels, respectively. An Andor iXon3 897 EMCCD camera (Andor Technology Ltd, Belfast, United Kingdom) was used to record images at a rate of 1 frame per 0.2–0.4 min in four neighboring spots. The experimental substrate was enclosed within a microfluidic chamber, and liquid sample was introduced at a flow rate of 100 μ l/min, as controlled by a peristaltic pump (model no. ISM833C, Ismatec, Wertheim, Germany). All measurements were conducted at room temperature (~25 °C).

6.2.6 Electrochemical Impedance Spectroscopy (EIS)

The EIS measurement platform consisted of gold electrodes that were supplied by SDx Tethered Membranes (Roseville, New South Wales, Australia) and coated with 10 mol % tether (benzyl disulfide octo-ethylene glycol phytanyl) and 90 mol % spacer (hydroxyl terminated benzyl disulfide tetra-ethylene glycol) molecules dispersed in ethanol. The assembled tethaPlate (SDx Tethered Membranes) contained six flow cells with a 2.1 mm² electrode surface area per cartridge and the formation of tethered bilayer lipid membranes (tBLMs) on the sensor surfaces was initiated by the addition of 8 μ l of 3 mM

DOPC lipid in ethanol. After 2 min incubation, the flow cell was rapidly rinsed with 10 mM Tris [pH 7.5, 150 mM NaCl] buffer (3 cycles $\times$ 100 μ l per cycle) in order to promote tBLM formation. Afterwards, 100 μ l of the appropriate test sample was injected into each well. TethaPod and tethaPatch units from SDx Tethered Membranes were used as the membrane conductance and capacitance reader and as the potentiostat connectivity unit, respectively. All measurements were collected under ambient room-temperature conditions ($\sim$ 25 $^{\circ}$ C) and analyzed using the tethaQuick software program (SDx Tethered Membranes), as previously described.²⁷⁻²⁸

6.2.7 Structural Modeling

The conformational properties of free peptide molecules in bulk solution were predicted by using the PEP-FOLD3 server, which is an online computational framework for *de novo* structural modeling.²⁹ The simulations were conducted without any preset data, *i.e.*, the modeling was unbiased. Structural prediction occurs in three consecutive steps: (1) the peptide structure is described by a series of fragments of four amino acids, overlapping by three amino acids each; (2) conformations are generated based on the prototype fragments followed by optional refinement with a Monte Carlo procedure; and (3) predicted conformations are ranked according to a scoring system.³⁰ The predicted conformational structures of free peptide monomers were visualized using the Swiss-PdbViewer program.³¹

6.3 Results and Discussion

6.3.1 Secondary Structure Characterization

In **Figures 6.1A** and **B**, the helix net projections and amino acid sequences of the AH and C5A peptides are presented and show the amphipathic disposition of each peptide, as indicated by polar (blue) and nonpolar (grey) faces. A small disturbance in the AH peptide's amphipathicity is observed due to polar residues being introduced near each end of the nonpolar face (S1 and K26 in N- and C-terminus respectively). The 18-mer C5A peptide is related to the middle section of the 27-mer AH peptide with 3 amino acids

differences that are highlighted in green. These differences represent conservative mutations that arise from substituting nonpolar with nonpolar (V8 to I6) or polar with polar (T14 to E12, T17 to S15) residues, and relate to the different genotypic origins of the two peptides as discussed in the Introduction. As such, both peptides have similar near-neutral electrical properties and the net charges of the AH and C5A peptides are 0 and -2 respectively. Likewise, the hydrophobicity (H) of each peptide was comparatively high (0.69 and 0.74, see **Table 6.1**). Hence, the primary amino acid sequences of the AH and C5A peptide are similar and endow the two peptides with similar physicochemical properties as well.

Next, lipid concentration-dependent CD spectroscopy experiments were conducted in order to measure the change in peptide secondary structure when peptide molecules partition from aqueous buffer solution into lipid membrane environments. This titration method also allowed us to identify how many lipid molecules are needed to interact with one peptide molecule in order to induce a stable α -helical conformation. Typically, amphipathic peptide molecules exhibit increased helicity in lipid membrane environments and the magnitude of the helicity change provides insight into the free energy of membrane partitioning.³²⁻³³ In the free state, both peptides exhibited partial α -helical structures in aqueous buffer solution due to the competition between intramolecular peptide hydrogen bonds and intermolecular binding to solvent molecules (**Figs. 6.1C,D**).³⁴ In lipid membrane environments, both peptides transitioned from a polar (solvent) to a nonpolar (lipid membrane) environment, and this transition coincided with a change in the peptide secondary structure from a partial helix to an increased α -helical conformation that was visible in the CD spectra. Helix stabilization is a common element of membrane-peptide interactions and the folded structure of a peptide molecule in a lipid membrane environment has a lower free energy (more stable) than the less-folded structure in bulk solution; the degree of helicity has also been shown to be related to the extent of permeabilization activity.³⁵⁻³⁷

Figure 6.1. Secondary structure comparison of AH and C5A peptides. Helical wheel projection of (A) AH and (B) C5A amino acid sequences showing an amphipathic distribution of hydrophilic (purple), positively charged (blue), negatively charged (red), and hydrophobic (yellow) amino acid residues. Circular dichroism (CD) spectroscopy analysis of (C) AH and (D) C5A peptides in the presence of lipid concentrations (with fixed peptide concentration of 100 μ M). Insets show the fractional helicity percentages of the two peptides as a function of normalized lipid concentration (defined as lipid concentration over peptide concentration; a value of 20 on the x-axis insets corresponds to 2000 μ M lipid, for example). All presented spectra were obtained by averaging three technical replicates, and the helicity percentages were calculated based on mean ellipticity values at the 222 nm wavelengths.

Table 6.1. Biophysical properties of AH and C5A peptides, including molecular weight (MW), isoelectric point (pI), net charge (Z), hydrophobicity (H), hydrophobic moment

(μH), and helical fraction ($f\text{H}$) are presented. The pI values were calculated from <https://pepcalc.com/>, Z, H, and μH were obtained from <http://heliquet.ipmc.cnrs.fr/cgi-bin/ComputParamsV3.py>, and $f\text{H}$ was measured by CD spectroscopy analysis. For $f\text{H}$ calculations, the measurements were conducted with 100 μM peptide in Tris buffer and in a lipid membrane environment with a P:L ratio of 1:12.

	MW	pI	Z	H	μH	$f\text{H}$ (%)
AH	3283.75	8.58	0	0.686	0.465	39.3 $\rightarrow$ 71.1
C5A	2310.63	4.32	-2	0.737	0.682	31.7 $\rightarrow$ 88.3

With increasing lipid concentration (in range of 0 to 2000 μM), the α -helical character of peptides was greater because more peptide molecules partitioned into the lipid membrane environment until saturation was reached. For AH peptide, the helicity increased from 39% in aqueous buffer to 71% at high lipid concentrations, and helix stabilization was reached at a peptide-to-lipid (P:L) ratio of about 1:10. By contrast, for C5A peptide, the helicity increased from 32% in aqueous buffer to 88% at high lipid concentrations, and helix stabilization was reached at a P:L ratio of about 1:6.

Together, the data indicate that the C5A peptide achieves more extensive helix stabilization in lipid membrane environments than AH peptide. C5A helix stabilization also occurs at a smaller P:L ratio, which is consistent with its greater membrane partitioning and the combination of these findings supports that the C5A peptide has greater interfacial activity against lipid membranes and is more potent than AH peptide at lower P:L ratios.

For both peptides, the ratio of the magnitudes of the two bands at the 208 and 222 nm wavelength positions, respectively (defined as $[\theta]_{222}/[\theta]_{208}$) were also investigated. Depending on the value of the ratio, it can be ascertained whether the peptides exist in a coiled-coil structure ($\gg 1$) or as non-interacting helices (≈ 1).³⁸ When AH peptide was in aqueous buffer, the ratio was 3.5, which indicates that peptide molecules exist as a coiled-coil structure (**Fig. 6.2**). In the presence of increasing lipid concentrations, the $[\theta]_{222}/[\theta]_{208}$ ratio decreased to around 1.1, which is more consistent with the value expected for non-interacting, linear helices rather than coiled-coil structures. By contrast,

the C5A peptide existed predominately as non-interacting monomers in buffer solution and the ratio reached ~ 1.0 in the presence of higher lipid concentrations. The results support that AH peptide transitions from a coiled-coil helical structure to a non-interacting, helical structure in the presence of lipid membrane environments while C5A peptide exists as non-interacting helices in both aqueous buffer and lipid membrane environments. Collectively, the findings support that AH and C5A peptides have distinct conformational changes associated with membrane partitioning and such factors help to explain why the two peptides have different potencies.

Figure 6.2. Ratio of θ_{222} and θ_{208} values for AH and C5A peptides as a function of normalized lipid concentration. The molar ellipticity ratio of $[\theta]_{222}/[\theta]_{208}$ indicates whether a peptide monomer exhibits predominately linear (single) or coiled-coil helical character. The data are plotted based on the CD spectroscopy results presented in Fig. 6.2.

6.3.2 Real-Time Tracking of Peptide-Induced Vesicle Rupture

To directly track membrane-peptide interactions, time-lapsed fluorescence microscopy experiments were conducted in a microfluidic chamber whereby peptide-mediated rupture of individual, tethered lipid vesicles on a functionalized glass surface was monitored in real-time. The vesicles were labeled with two fluorescence probes: a water-soluble calcein dye (green) was encapsulated in the vesicle interior and a rhodamine dye-labeled phospholipid Rh-PE (red) was incorporated into the vesicle bilayer in order to track membrane permeabilization and lysis, respectively. The two types of membrane-interaction events were detected on the single-vesicle level based on monitoring decreases in the intensity of the two fluorescence signals per individual vesicle and over 300 vesicles could be monitored simultaneously per experiment.

For small vesicles (< 125 nm diameter), 100 nM AH peptide ruptured nearly all vesicles within around 12 min while 100 nM C5A peptide ruptured all vesicles within only 3 min (**Fig. 6.3A**). By contrast, for larger vesicles (> 125 nm diameter), 100 nM AH peptide only ruptured a minor fraction of vesicles and most tethered vesicles remained intact (**Fig. 6.3B**). On the other hand, 100 nM C5A peptide was still active against larger vesicles and ruptured them within 3 min. At 10 nM peptide concentration, AH peptide lost membrane-disruptive activity against even small vesicles (< 125 nm diameter) whereas C5A peptide maintained similar activity levels and caused vesicle rupture within ~10 min (**Fig. 6.3C**).

The final intensity values of the rhodamine signal per individual vesicle versus each individual vesicle's size were plotted in order to quantitatively determine what fraction of vesicles was "ruptured" in different size categories.³⁹ For 100 nM AH peptide, 92.8% of small vesicles (< 125 nm diameter) were ruptured while only 18.0% of larger vesicles were ruptured (> 125 nm diameter) (**Fig. 6.3D**). This vesicle size-dependent trend contrasted with the results obtained with 100 nM C5A peptide, in which case the peptide ruptured 100% of vesicles across the tested range of vesicle sizes. Strikingly, 10 nM AH peptide displayed negligible activity (0.2% rupture efficiency) against small vesicles (< 125 nm diameter) whereas 10 nM C5A peptide still exhibited 100% rupture efficiency (**Fig. 6.3E**).

Figure 6.3. Distinct membrane-disruptive potencies of AH and C5A peptides. Time-lapsed fluorescence microscopy images of encapsulated calcein (green, top) and Rh-PE (red, bottom) dye probes for addition of 100 nM peptide to (A) small vesicles (< 125 nm diameter) and (B) large vesicles (> 125 nm diameter), or (C) 10 nM peptide to small vesicles (< 125 nm diameter). Scale bars are 10 μ m. (D-E) Corresponding single-vesicle data of peptide-induced vesicle rupture as a function of vesicle size, based on the rhodamine signal. Rupture is defined as when the final fluorescence intensity value of an individual vesicle was 30%

or less of the original fluorescence intensity value of that vesicle. Each data point represents an individual vesicle.

Figure 6.4. Change in normalized fluorescence intensity of individual, tethered vesicles as a result of membrane-peptide interactions. (A-F) Top: Representative profiles of single-vesicle rupture kinetics for AH and C5A peptides at different peptide concentrations and for different vesicle size ranges. Green and red colors denote calcein and rhodamine signals, respectively. Bottom: Corresponding histogram statistics of membrane permeabilization (green) and membrane lysis (red) time scales, where applicable.

Quantitative analysis of the vesicle rupture kinetics further confirmed that C5A peptide is more potent (ruptures lipid vesicles at a 10-fold lower bulk peptide concentration than AH) and causes membrane permeation and lysis on quicker time scales than AH peptide. Treatment with 100 nM AH peptide caused rapid permeabilization of small

vesicles in around 2 min while membrane lysis typically occurred within 5 min (**Fig. 6.4A**). However, 100 nM AH peptide caused much slower permeabilization of larger vesicles without inducing membrane lysis (**Fig. 6.4B**). Likewise, 10 nM AH peptide caused membrane permeabilization of smaller vesicles but did not cause membrane lysis (**Fig. 6.4C**). As such, AH peptide-mediated pore formation is selective for small vesicles while a relatively high bulk peptide concentration is required to cause membrane lysis. By contrast, 100 nM C5A peptide is able to rapidly permeabilize and lyse both small and large vesicles on similar time scales of around 2 min (**Figs. 6.4D,E**). Nearly identical results were obtained when 10 nM C5A peptide was added to small vesicles as well (**Fig. 6.4F**). Thus, C5A peptide exhibits more potent, indiscriminate activity against lipid vesicles, as indicated by vesicle-independent rupture activity and rapid kinetics. Of particular note, the time scales of membrane permeabilization and membrane lysis were nearly identical for C5A peptide, suggesting that its membrane-interaction profile is distinct from that of AH peptide which induces membrane lysis *after* a critical density of pores is formed. On the other hand, AH peptide exhibits vesicle size-dependent rupture activity and the rate of membrane permeabilization is vesicle size-dependent as well, which is consistent with its known membrane curvature-sensitive, pore-forming behavior.

6.3.3 Peptide-Induced Membrane Leakage

To further investigate the effects of AH and C5A peptide treatment on membrane permeability, electrochemical impedance spectroscopy (EIS) measurements were conducted using a tethered bilayer lipid membrane (tBLM) platform. A schematic illustration of the measurement setup is presented in **Figure 6.5A**, and the experimental objective was to detect membrane permeabilization based on ion leakage across the lipid bilayer. The setup measures the electrical conductance (G_m) across the tBLM, which is the inverse of its electrical resistance and expressed as $G_m = I/V$, where I and V are the current and voltage, respectively. The setup also measures the capacitance (C_m) of the tBLM, which signifies the magnitude of charged ions that are stored across the lipid bilayer and is expressed as $C_m = q/V$, where q and V are the charge and voltage, respectively. When membrane leakage occurs, the G_m and C_m values both increase – larger increases

indicate greater membrane leakage – and a well-sealed tBLM is important for baseline stabilization. In this experiment, the tBLM was formed by rapid solvent exchange and the initial G_m and C_m values were less than 2 μS and around 15–25 nF, respectively, which indicated good sealing properties.⁴⁰

Figure 6.5. Electrochemical impedance spectroscopy analysis of membrane-peptide interactions involving AH and C5A peptides. (A) Schematic illustration of measurement setup. Peptide concentration-dependent changes in G_m (top) and C_m (bottom) signals as a function of time for (B) AH and (C) C5A peptides, respectively. Peptides were added at approximately $t = 20$ min.

Figures 6.5B and C present the concentration-dependent measurements results for AH and C5A peptides, respectively. Both peptides showed concentration-dependent membrane leakage activity, with distinct differences in the level of membrane

permeabilization. At the highest test concentration, 8 μM AH peptide caused stable G_m and C_m shifts of around 40 μS and 60 nF, respectively. In marked contrast, 8 μM C5A peptide caused appreciably larger G_m and C_m shifts of around 550 μS and 270 nF, respectively, and the C_m shift continued to increase over the measurement time period. The distinct membrane-interaction profiles are consistent with AH peptide causing membrane thinning⁴¹ while C5A peptide exhibited more indiscriminate, membrane solubilization, which bore resemblance to the behavior of sodium dodecyl sulfate (SDS) surfactant in the order of magnitude, but with slower kinetics based on the previous experience. Together with the foregoing experimental data, the EIS results indicate that AH and C5A peptide have distinct membrane-interaction profiles that arise from unique conformational properties.

6.3.4 Structural Modeling of Peptide Conformations

To further evaluate the conformational properties of the two peptides, their secondary structures were modeled by performing computational simulations with the PEP-FOLD online server.⁴²⁻⁴³ **Figure 6.6A** presents the simulated probabilities that the different amino positions are in helix, extended, or random conformations, as indicated by red, blue, and green colors, respectively. In general, it was predicted that both ends of the AH peptide have a high probability of helical conformation while it was predicted that the middle region of AH peptide (W9–18D) is likely non-helical. On the other hand, the simulation results predicted that the C5A peptide has more uniform helical character throughout its amino acid sequence. Based on these predictions, the highest-ranking conformers of AH peptide mainly consisted of two helical regions that are separated by a non-helical hinge in the middle (**Fig. 6.6B**). This finding is consistent with the CD spectroscopy results indicating that the AH peptide likely has a coiled-coil structure, whereby the two helical regions of a peptide monomer interact and stabilize one another while the non-helical hinge supports conformational freedom to minimize the peptide free energy. By contrast, the highest-ranking conformers of the C5A peptide have linear helix structures, which also agrees well with the CD spectroscopy results.

Figure 6.6. Structural modeling of AH and C5A peptides and implications for targeting selectivity. (A) Simulated probabilities of helical (red), extended (blue), and random (green)

conformations are shown for each amino acid position in the peptide sequences. (B) Highest-ranking conformers of AH and C5A peptides are presented, pointing to distinct conformational properties of the two peptides in bulk solution. (C) Schematic illustration of how AH and C5A peptides interact with lipid membranes. Elements are not drawn to scale.

Taking into account the experimental and simulation results, these findings support that AH and C5A peptides exhibit distinct conformational properties that influence their membrane-disruptive activities, as presented in **Figure 6.6C**. While AH peptide exhibits some degree of conformational flexibility in bulk solution, the simulation results indicate that it likely has two helical segments bifurcated by a flexible hinge and this conformational arrangement offers insight into its empirically observed membrane curvature sensing properties. In high-curvature lipid membranes, there can be hydrophobic defects between neighboring lipid molecules⁴⁴ and it appears that the AH peptide conformation is well-suited for fitting within these defects.

On the other hand, free C5A monomers exhibit lower helical character in the bulk solution while demonstrating greater membrane partitioning, as indicated by helix stabilization in the presence of lower total lipid concentrations in the CD spectroscopy experiments. As a result, C5A peptide undergoes more extensive conformational changes – marked by a significant increase in helical character – upon membrane partitioning, which drives higher levels of interfacial activity.^{37, 45}

6.4 Conclusions

As such, C5A peptide is less discriminate of lipid membrane properties and there is a larger thermodynamic driving force to favor membrane partitioning. This finding explains why C5A peptide is more potent than AH peptide, and interacts with lipid membrane via a distinct mechanism that is more akin to membrane solubilization than the curvature-sensitive pore formation exhibited by AH peptide. Collectively, these findings reveal how the conformational properties of AH and C5A peptides influence structure-function relationships and help to explain the distinct membrane interaction profiles of the two antiviral peptides. From a structural perspective, such information also provides a starting

point to engineer antiviral peptides with desirable conformational properties and to screen candidate amino acid sequences using *in silico* methods and surface-sensitive measurement strategies. Looking forward, it will also be useful to apply this integrative approach across experiment and computational simulation to characterize the interactions of membrane-active peptides with complex lipid membrane models and in conjunction with other surface-sensitive measurement techniques as well.⁴⁶⁻⁴⁷

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Chapter 7 Conclusion and Future Work

This chapter highlights the important outcomes and conclusions achieved from this project, and demonstrates significant examination based on scientific approaches that could contribute to develop an analytical framework for characterizing antiviral agents. Based on the findings, further optimization of lead template sequence and in vitro/in vivo studies are introduced as future work to highlight how the insights gained from this thesis can be translated to improvement of antiviral strategies. Looking forward, the measurement capabilities developed in this work will be broadly applicable to studying other classes of membrane-active agents, including small molecules, additional peptides, and enzymes, as well.

7.1 Conclusion and Discussion

In this thesis, the integrative approach across model membrane platforms and corresponding surface-sensitive measurement techniques were used and developed to understand how lipid envelope antiviral disruption (LEAD) antivirals interact with membranes. A particular attention was drawn to the lead compound, AH peptide, to establish comprehensive analytical framework to characterize the molecular structure with association of lipids, to obtain the membrane interaction profiles, and to understand the relationship between structure-function. The development of an antiviral peptide-screening platform integrates soft matter design components together with biomaterial and surface functionalization strategies to facilitate highly parallel measurements tracking peptide-induced destabilization of nanoscale, virus-mimicking small unilamellar vesicles with tunable size and composition. The key design steps were presented, including tethering strategy, measurement concept, and data analysis, and important advantages of this single-particle tracking scheme were discussed.

The first aim to improve LEAD agent screening platform design and data analysis was presented in Chapter 4. The improvement was based on the identification of the technical artifacts (dye leakage and photobleaching) and modulation of membrane composition and corrective measurements. The results demonstrate a broadly applicable analytical framework to consider artifacts and improve measurement accuracy in single lipid vesicle arrays. In the next chapter, the second aim is described addressing the development of virus-mimetic clusters of tethered vesicles that can model the architectural configuration of clustered viral particles, which was accomplished by incorporating micropatterning strategy. The platform was also compared with the conventional single vesicle platform to observe the difference in kinetics. The micropatterning strategy applied in here was proven highly effective and the measurement capabilities will be advantageous for the exploration of membrane factors including various lipid types. Last aim was presented in Chapter 6 to compare two antiviral peptides by adopting an integrative approach with diverse types of model membranes to investigate the different activities of and further draw structure-function relationships. In summary, this work will improve the knowledge about the design principles behind membrane-active, antiviral peptides and lead

to the identification of promising antiviral drug candidates to advance to biological evaluation. Successful incorporation of this approach to fabricate tethered lipid vesicle platform would potentially enable mapping drugs' structure-function relationships. Mostly in this thesis, single-component zwitterionic DOPC lipids was mainly used to present a simplified and well-controlled model. For future studies, other lipid compositions and increasing complexity would benefit scrutinizing the effect of membrane-drug interaction. Looking forward, the measurement capabilities developed in this work will be broadly applicable to studying other classes of membrane-active agents, including small molecules, additional peptides, and enzymes, as well.

7.2 Future Outlook

7.2.1 Optimization of the Lead Template Sequence

Based on the previous reports and this thesis work, AH peptide has been proven to preferentially target of high-curvature lipid membranes with distinct membrane-interaction profiles. As a lipid envelope antiviral disruption (LEAD) template, various mutations (e.g., enantiomers, truncation, and point variations) in AH peptide sequence or structure might confer different targeting selectivity and potency that offer the opportunities of tuned antiviral activities. Furthermore, modulations in the peptide sequence based on rationale design can reveal the structure-function relationships of peptides that will be useful to apply in drug development efforts.

L (levorotary)- and D (dextrorotary)-isomeric forms

The amino acids found in nature commonly exist in L (levorotary)-isomeric forms whereas D (dextrorotary)-isomeric forms are found in rare cases such as peptidoglycan cell walls or antibiotics. As most of biological organism is consist of L-forms of homochiral amino acids, L- peptides are much more susceptible to degradation by proteases (i.e., low bioavailability), which is a major limiting factor of peptide application in therapeutics. Therefore, peptides tend to have low intravenous bioavailability as well as poor oral

bioavailability, requiring frequent injections. The D-isomeric forms of peptides, on the other hand, have been reported that they can improve the stability and bioavailability. Inspired by these advantages of D-enantiomer, one of the previous study has utilized AH peptide composed of D-enantiomer. Interestingly, D-enantiomer of AH peptide (AH-D) showed not only higher stability but more potent activity towards rupturing vesicles compared to L-enantiomer of AH peptide (AH-L), which is a conventional sequence that were used as a template (Fig. 7.1A). The circular dichroism analysis in deionized water showed that the spectra of the two enantiomers were mirror images as expected (Fig. 7.1B).

Figure 7.1 Characterization of AH peptide enantiomers. (A) Quartz crystal microbalance with dissipation (QCM-D) measurements of AH peptide-induced rupture of surface-adsorbed vesicles.

(B) Circular dichroism spectra of AH peptide enantiomers in deionized water, exhibiting mirror images.

Then, single vesicle assay was performed with membrane-embedded Rh-PE only and AH-D peptide induced efficient vesicle rupture from 50 nM with much quicker response than AH-L (Fig. 7.2A). With increasing AH peptide concentration (100 and 1000 nM), the timescale of peptide-induced vesicle rupture decreased with maintaining the trend that AH-D always induced quicker vesicle rupture than AH-L peptide (Fig. 7.2B and C). The analysis on the effect of membrane curvature by adding 100 nM AH-D and AH-L peptide showed that as potent AH-D peptide is, it was less size-discriminative than AH-L peptide (Fig. 7.2D). Although both peptides preferentially targets smaller vesicles, as indicated by more extensive rupture, 100 nM AH-D peptide targeted vesicles around 300 nm diameter and smaller, while 100 nM AH-L peptide targeted ones below 100 nm (Fig. 7.2E). Therefore, these results indicate that the size targeting ability can be tuned and governed by one or more biophysical factors that these peptides possess, which open the door for further characterizations. For example, circular dichroism measurements with varied sizes of vesicles or peptide-to-lipid ratio might reveal distinct membrane-interaction profiles. Also, double-dye system with calcein in single vesicle assay will be beneficial to scrutinize pore-forming behavior of both peptides.

Figure 7.2 Single vesicle assay results of AH peptide enantiomers. (A-C) Right: The F.I. of individual vesicles were tracked. Left: Histogram of lag time taken before the rupture of vesicles.

(D) Quantification of peptide-induced vesicle rupture as a function of vesicle diameter, where rupture is characterized by the final fluorescence intensity value of individual liposomes after treatment with 100 nM AH-D or AH-L peptide. Each symbol represents data from an individual vesicle. (E) Fraction of ruptured liposomes in different size categories based on the data in (D). An individual liposome is classified as “ruptured” when its final fluorescence intensity is 30% or less of the original fluorescence intensity.¹

Truncated forms

Several studies have shown that it is possible to considerably reduce the size of peptides and retain antimicrobial activity. The reduced sizes or truncated forms are not only beneficial to simplify the synthesis process but also they aid our understanding of mechanism. For example, dermaseptin (originally, 34-amino acid residue) is an antimicrobial peptide that is known to inhibit the growth of pathogenic fungi. The shortened analogues exhibited different activities depending on the length and location. By shortening the sequence from the NH₂-terminal (*e.g.*, remaining 14-34, 16-34, 20-34, and 28-34 amino acids) showed the loss of antimicrobial activity whereas lengthening the sequence from amino acids 1-4 to 1-8 and 1-18 resulted in a gradual recovery of the activity of the lead sequence. By repeating this type of analysis, the authors concluded that the peptide sequences responsible for the antimicrobial activity of dermaseptin are amino acids 1-18 from NH₂-terminal.²

Similarly, C5A peptide is a truncated analogue of AH peptide, considering that both peptides are derived from the same region of the protein (NS5A of HCV) within close genotype variations (Table 7.1). C5A peptide has perfect hydrophobic and hydrophilic face divided; leading to higher potential of pronounced α -helicity. On the other hand, the beginning and ending sequences of AH peptide violate the amphipathicity, resulting in hydrophilic moieties intervening the hydrophobic sides. Although the extent of helicity does not fully govern the potency of membrane activity, truncation of certain sequence can lead to higher stability or helicity, thus can participate in tuning the membrane interaction profiles. In addition, considering that the C5A peptide has higher cell toxicity than AH peptide due to its potency, intended breakage of helicity might aid this objective. One of

the most broadly studied peptides, melittin, was reported that the segment between 1-20 is membrane active but is not lytic, and the cationic segment 21-26 is inactive.³ By contrast, the segment of 11 residues between 12-22 is known to be ‘surface seeking’ region,⁴ whereby most of them exhibit antimicrobial and hemolytic activities. Following this observation, one of the successful version of truncated melittin can be MCF peptide, which is based on the melittin C-terminus (Table 1). It exhibits ~5 times less antibacterial activity compared to the parent melittin but also, 300 times less hemolytic.⁵

Table 7.1 Lead peptide sequences and their truncated or point mutated analogues that show different activities towards membrane interaction profiles as well as antiviral or antibacterial activities.

	Amino Acid Sequence																											
	N-term.	5					10					15					20					25					C-term.	
AH	S	G	S	W	L	R	D	V	W	D	W	I	C	T	V	L	T	D	F	K	T	W	L	Q	S	K	L	
C5A		S	W	L	R	D	I	W	D	W	I	C	E	V	L	S	D	F	K									
Melittin	G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	R	K	R	Q	Q		
MCF													G	L	P	A	L	I	S	W	I	K	R	K	R	Q	Q	G
MCFA													G	L	K	K	L	I	S	W	I	A	A	K	R	Q	Q	G
Point variation							G		A	L			S		G							Q	Q	Q	Q		L	
													A									A	A	A	A			
Mel-P5	G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	K	A	A	Q	Q	L		

Blue Letter: Conservative mutation

Red Letter: Non-conservative mutation

Point variations

Followed by truncated analogues, point mutations are also powerful strategy to optimize the lead template’s activity. For example, abovementioned MCF peptide was further modified into MCFA, whereby two cationic residues were swapped (the N-terminal part from the C-terminal part), exhibiting antibacterial activity comparable to that of melittin (higher than MCF) with the similar hemolytic effect to MCF. This mutation was rationally designed, as proline residue is known to break the secondary structure of peptides, thus replacing it to lysine would aid the stability without dramatic alteration of overall

hydrophobicity. Therefore, it is possible to design short peptides with relatively selective anti-infective activity by judiciously replacing the certain amino acids. Furthermore, this strategy has been proven highly effective in recent studies of high-throughput screening.⁶

⁷ First, combinatorial peptide library was created by varying a number of critical residues in melittin template (Table 1, 'point variation'). Then, all peptides underwent high-throughput assay to assess the potency of peptide-induced vesicle pore formations within the peptide-to-lipid ratio, where the original melittin is not active. As a result, 0.1% of the library sequences including Mel-P5 were found to be significantly more active than melittin. The screening based on rational design can provide the necessary factors that can be functional membrane-active peptides such as positioning of certain amino acids and overall amphipathicity based on helical net diagram.

7.2.2 Preclinical Studies

After establishing the promising antiviral agents based on model membrane screening, the next step would be biological investigations involving *in vitro* tests and *in vivo* animal studies. First, cell viability tests to check whether the drug candidate can inhibit the virus-induced cell death. In one of the main study with AH-D peptide, primary neuronal cells were infected by Zika virus and the peptide was treated after as long as 48 h of infection (Figure 7.3A). The post-infection tests provide inhibitory effect (reducing viral loads by suppressing virus replication) and cell viability of the candidate drug. The results indicated that 10 nM or higher peptide treatment effectively prevented cell death and up to 500 nM was non-toxic to uninfected cells. In addition, there is an *in vitro* plaque reduction neutralization test (PRNT) to measure the plaque forming efficiency of a virus in the presence of different concentrations of antiviral agents (Figure 7.3B). Especially for flaviviruses, this assay is considered as the gold standard for the detection although the test itself is labor and resource intensive. The 50% inhibitory concentration (IC₅₀) values obtained by this assay is efficient to compare the potency of drugs depending on the virus strains.

Figure 7.3 AH-D peptide exhibits inhibitory effect in primary neuronal cells and in vitro antiviral activity. (A) Left: Effect of AH-D peptide on viability of ZIKV HS-2015-BA-01-infected cells.

Right: Production of infectious virus from infected cells. Inhibitory activity of AH-D peptide against viral infection in Vero cells, reported by 50% inhibitory concentration (IC_{50}) values.¹

Next, animal model studies are considered essential to translate the bench-side drugs to bedside therapeutics. However, small animals such as mice are often not the natural host of human-infecting viruses. Therefore, certain virus strains and the corresponding type of animal models should be carefully chosen or adapted to each case in order to produce meaningful clinical signs and data.^{8,9} For example, (partially) immunocompromised mice or severe combined immune deficiency (SCID) mice transplanted with human cells are common types of models that can be used to study dengue virus infections.¹⁰ As for the recent study involving AH-D peptide and Zika virus infection, type I interferon receptor deficient mice ($A129^{-/-}$, $IFN-\alpha/\beta R^{-/-}$) were inoculated intravenously with 4×10^3 plaque-forming units (PFU) of the pathogen, which caused death after 7 days when the mice were not treated.¹ The therapy started after three days to set the treatment more relevant to the reality while 10 out of 12 mice were protected. This particular model was selected because ZIKV infection showed leukocytosis and increased level of brain inflammatory markers and intraocular eye pressure, which are similar clinical symptoms as humans.¹¹ Therefore, change in body mass, intraocular pressure and leukocytes level were tracked daily (Figure 7.4A). The therapeutic effect can be also evaluated by the measurements of viral loads in body parts/organs (e.g., serum, spleen, brain, and nerves) (Figure 7.4B) and the levels of cytokines as both values should be dropped if the therapy works (Figure 7.5A). Furthermore, histopathological data using microscopy can be examined to manifest the virus-specific damages. For instance in ZIKV case, cerebral damage and neuroinflammation/degeneration can be further investigated by microscopy images of various brain sections (Figure 7.5B). In addition, direct assessment of antiviral activity of AH-D peptide in brain demonstrated that the peptide could penetrate the blood-brain-barrier (BBB) (Figure 7.5C). Looking forward, the biological results highlight the opportunities afforded by linking biophysical assessments using model membranes and activity profiling.

Figure 7.5 Assessments of therapeutic activity of AH-D peptide in mice.¹ (A) One of cytokine molecules, TNF- α and CCL5, concentrations in brain on day 7 post-infection. (B) Histopathological scores from hematoxylin and eosin (H&E) staining of brain sections. (C) Unidirectional blood-to-brain influx rate.

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List of Publications

Directly relevant to this thesis:

1. **Soohyun Park**, Jackman JA, Xu XB, Weiss PS, Cho NJ. “Micropatterned Viral Membrane Clusters for Antiviral Drug Evaluation.” *ACS Applied Materials & Interfaces* 11(15), 13984–13990 (2019).
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Others:

6. Teng-Fei Fan*, **Soohyun Park***, Shi Q, Zhang X, Liu Q, Song Y, Chin H, Ibrahim MS, Mokrzecka N, Yang Y, Li H, Song J, Suresh S, Cho NJ. “Transformation of Hard Pollen into Soft Matter.” *Nature Communications* 11, 1449 (2020). *denotes equal first authors.

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