Molecular Recognition at the Membrane

Dissertation

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By

Yun Gong, B.S.

Chemistry Graduate Program

The Ohio State University

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Dissertation Committee:

Dr. Dennis Bong, Advisor

Dr. Ross E. Dalbey

Dr. Jon Parquette
“Second star to the right, straight on till morning.”

- *Peter Pan*
ABSTRACT

In two separate systems, we have demonstrated selective vesicle fusion with biological recognition motifs not natively associated with lipid bilayer fusion, thus broadening the scope of recognition-guided membrane activation. These findings have resonance with goals in targeted chemical delivery and nanoscale compartmentalized chemistry.

Our first system employs two complimentary surface bound fusogens: vancomycin glycopeptide coupled to the antimicrobial peptide magainin II, and D-Ala-D-Ala-OH dipeptide coupled to a phospholipid derivative. Fusion was characterized by dynamic light scattering and FRET experiments with lipid bound fluorophores. We have demonstrated that appropriately designed membrane anchored molecular recognition motifs have the ability to activate specific membrane merging. We further characterized this fusion reaction with regard to the following variables: effects of fusogen concentration, lipid composition, and membrane charge. The results indicate that these parameters are determinants of fusion rate, vesicle stability, peptide binding, catalytic fusion, and membrane disruption during fusion. Notably, these data highlight the importance of coupling between molecular recognition and insertion for bilayer activation as well as the critical role of membrane subdomain formation for membrane fusion reactivity. The phenomena are general to lipid membrane chemistry, and
therefore our findings provide a guideline for understanding more complex biomembrane systems.

In our second fusion system, cyanuric acid (CA) and melamine (M) functionalized lipids in membranes exhibited robust hydrogen-bond driven surface recognition in water, facilitated by multivalent surface clustering of recognition groups and variable hydration at the lipid-water interface. We introduced a minimal recognition cluster: three CA or M recognition groups were forced into proximity by covalent attachment to a single lipid headgroup. This trivalent lipid system guides recognition at the lipid-water interface using cyanurate-melamine hydrogen bonding when incorporated at 0.1-5 mole percent in fluid phospholipid membranes, inducing both vesicle-vesicle binding and membrane fusion. Fusion was accelerated when the antimicrobial peptide magainin was used to anchor trivalent recognition, or when added exogenously to a pre-assembled lipid vesicle complex, underscoring the importance of coupling recognition with membrane disruption in membrane fusion. Membrane apposition and fusion were studied in vesicle suspensions using light scattering, FRET assays for lipid mixing, surface plasmon resonance and cryo-electron microscopy. Recognition was found to be highly spatially selective as judged by vesicular adhesion to surface patterned supported lipid bilayers (SLBs). Fusion to SLBs was also readily observed by fluorescence microscopy. Together, these studies indicate effective and functional recognition of trivalent phospholipids, despite low concentration, and solvent competition for hydrogen bond donor/acceptor sites. This novel designed molecular recognition motif may be useful for directing aqueous-phase assembly and biomolecular interactions.
Lastly, we extend our study to other molecular recognition driven events such as phagocytosis. Preliminary experiments are carried out in regard to the affinity labeling of phosphatidylserine receptors in cell lysates and the design of phosphatidylserine modified polymers as mimics of apoptotic cells. Current results on affinity labeling of PS-receptors suggests possible proteins that are selectively labeled but they need to be further confirmed by mass spectroscopy. While uptake of the first generation of PS-polymers with FITC tags is successful, subsequent cytokine production still needs to be verified, and better controls need to be applied.
To my family
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VITA

October 07, 1982………………………………………….Born, Changsha, Hunan, China
2000………………………………………………………Shenzhen Middle School, China
2004……………………………………………………..BS, Chemistry, Nanjing University, China
2004……………………………………………………..Graduate Student Summer Fellowship
2004-2007………………………………………………….Graduate Teaching Assistant
2007-2009……………………………………………………Graduate Research Assistant

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LIST OF ABBREVIATIONS

AAA+ ATPases associated with various cellular activities
ABPP activity based protein profiling
ACN Acetonitrile
ANTS 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt
BP Benzophenone
AFF-1 anchor cell fusion failure-1
Ala Alanine
AMPs antimicrobial peptides
amu atomic mass unit
ATP adenosine triphosphate
CA cyanuric acid
Cbf Carboxyfluorescein
CD circular dichorism
cryo-TEM cryo-electron microscopy
DHPE 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine
DIC diisopropyl carbodiimide
DIEA N,N-Diisopropylethylamine
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<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPA</td>
<td>dipicolinic acid</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered Salines</td>
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<tr>
<td>DPPC</td>
<td>dipalmitoyl phosphatidyl choline</td>
</tr>
<tr>
<td>DPX</td>
<td>p-xylene-bis-pyridinium bromide</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EFF-1</td>
<td>epithelial fusion failure-1</td>
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<tr>
<td>eggPC</td>
<td>L-(\alpha)-lysophosphatidylcholine (Egg, Chicken)</td>
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<tr>
<td>ESI</td>
<td>electrospray ionization</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
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<td>FRET</td>
<td>förster resonance energy transfer</td>
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<tr>
<td>HB</td>
<td>hydrogen-bonding</td>
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<td>HBSS</td>
<td>Hanks' balanced salt solutions</td>
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<tr>
<td>HOBt</td>
<td>N-hydroxybenzotriazole</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>HRP</td>
<td>horse radish Peroxidase</td>
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<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
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<tr>
<td>Kaa</td>
<td>Lys-D-Ala-D-Ala</td>
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LUVs  large unilamellar vesicles
M  melamine
MALDI  matrix-assisted laser desorption/ionization
M.W.  molecular weight
NBD  \( N-(7\text{-nitrobenz}-2\text{-oxa}-1,3\text{-diazol}-4\text{-yl}) \)
NMP  N-methylpyrrolidinone
NSF  N-ethylmaleimide-sensitive factor
OG  oregon green
PDMS  poly(dimethylsiloxane)
PEG  polyethylene glycol
PMA  phorbol-12-myristate-13-acetate
POPC  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPE  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
POPG  1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1\text{'-rac-glycerol})
POPS  1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
PS  phosphatidylserine
Rh  rhodamine
RT  room temperature
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLB  supported lipid bilayer
SNAP  soluble NSF attachment proteins
SNAREs  soluble N-ethylmaleimide-sensitive factor attachment protein receptor
<table>
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<th>Acronym</th>
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<tr>
<td>TBTU</td>
<td>O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor beta-1</td>
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<tr>
<td>TR</td>
<td>texas red</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>t-SNAREs</td>
<td>target membrane SNAREs</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
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<tr>
<td>v-SNAREs</td>
<td>vesicle membrane SNAREs</td>
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PREFACE

Molecular recognition is one of the most important processes in biological assemblies. Recognition events at interfacial environments such as membrane surfaces provide great insights into a broad range of biological phenomena such as membrane fusion and fission, cellular turnover, antibody binding, and protein translocation across membranes. In this thesis, we focus on two molecular recognition driven processes: membrane fusion and phagocytosis. The study of membrane fusion can not only help us understand native fusion events but also has great impact on targeted liposomal drug delivery strategies. Phosphatidylserine and receptor interactions play a critical role in the progress of phagocytosis. Our development of phosphatidylserine affinity probes would provide an unbiased screen method for surface receptors, while our synthetic phosphatidylserine bearing polymers could open a door to novel anti-inflammatory drugs.

Chapter 1 provides a general introduction of membrane fusion, while Chapter 2 and 3 discuss our vancomycin-D-Ala-D-Ala recognition based fusion systems in details. Chapter 4 introduces a second fusion system driven by cyanuric acid and melamine binding. In Chapter 5, a brief introduction about phagocytosis and phosphatidylserine recognition is followed by preliminary results carried out in this project. Chapter 7 includes some useful protocols involved throughout this research.
CHAPTER 1

INTRODUCTION

1.1. Membrane Fusion and Activation

1.1.1. Membrane Fusion

Membrane fusion occurs when two individual membranes merge into one\textsuperscript{1,2}. This process has attracted much attention from researchers, not only because it is a fundamental biological process in life, but also it shows great potential in liposomal cargo delivery into the cells.

Native fusion reactions, usually sorted as viral fusion, intracellular fusion, mitochondrial fusion and cell-cell fusion, have been extensively studied but not completely understood. The fusion mechanism is conserved within the same category while vastly distinct across different ones. Several approaches are used to understand membrane fusion: genetic screens for genes that are relevant to the process; investigation of related proteins and their binding interactions; reconstitution of fusion proteins into liposome systems to identify their functions.

1.1.2. Viral Fusion

In the case of viral fusion, viral fusion proteins mediate all steps of fusion. They are previously classified as class I, class II and class III viral fusion protein\textsuperscript{3}, with
representatives of each class: influenza4, dengue5,6 and vesicular stomatitis viruses7,8, respectively. But as this classification now obscures as much as it clarifies, it is avoided in some reviews9. Although viral fusion proteins vary greatly in structure, the common mechanism involves a large-scale conformational change in the fusion protein coupled to apposition and merger of the two bilayers. It can be defined as five steps. Step 1 (Figure 1.1b): The protein becomes the bridge between the two bilayers and forms the extended intermediate. Step 2 (Figure 1.1c): The bridge collapses and brings the two membranes together. Step 3 (Figure 1.1d): The energy barrier lowers upon the distortion of the membranes so that a hemi-fusion stalk forms. Step 4 (Figure 1.1e): The hemi-fusion stalk opens to form a transient fusion pore. Protein refolding causes the irreversible formation of fusion pore, which then expands in the final step to complete the fusion event.

![Sequence of events in membrane fusion promoted by a viral fusion protein.](image)

**Fig 1.1.** Sequence of events in membrane fusion promoted by a viral fusion protein. Figure adapted from reference 9.

### 1.1.3. Intracellular Fusion by SNAREs

For intra-cellular fusion processes, the SNAREs10-15 (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein superfamily and its associated
proteins (SM proteins, complexin, NSF, Sec18p and others) have been recognized as the core component of protein complexes that drive intracellular vesicle fusion by the formation of a parallel four helical bundle. Most SNAREs have a 60-70 amino acids long conserved SNARE motif within hepta-repeats, with a single transmembrane domain connected to the C-terminal by a short linker and an N-terminal folded domain varying between different types of SNARES, although some subset of SNAREs lack N-terminal domain or transmembrane domains. The SNARE motif was originally classified as v-SNAREs (vesicle membrane SNAREs) and t-SNAREs(target membrane SNAREs). However, it was later found that certain SNAREs function with various partners in different transport steps in the case of homotypic fusion reactions. From a more in-depth understanding of SNARE complexes, a more rigorous classification defines each α-helical protein from the hetero-oligomeric, parallel four-helical bundle as Qa-, Qb-, Qc- and R-SNAREs, which is based on the fact that the center of the bundle contains 16 largely hydrophobic stacked layers of interacting side chains with an exceptional ‘0’ layer that contains three highly conserved glutamine(Q) residues and one arginine(R) residue, accordingly (Figure 1.2). For example, in neuronal SNARE core complex, SNARE motifs contains syntaxin-1(t-SNAREs or Qa), SNAP-25(t-SNAREs or Qbc), and VAMP(v-SNARES or R), in which SNAP-25 is composed of two different SNARE motifs that are joined by a flexible linker. SNARE motifs can also interact in other combinations that are less stable, such as Qaaaa, Qabab, or even an anti-parallel QabcR complex due to their amphiphilic nature.
**Fig. 1.2.** SNARE core complexes. (a) A crystal structure of the neuronal SNARE core complex. This complex contains the SNARE motifs of syntaxin-1 (Qa; red), SNAP-25 (25-kDa synaptosome-associated protein; Qb and Qc; both green), and VAMP (vesicle-associated membrane protein)/synaptobrevin (R; blue). The C-terminal ends of the helices, which all point towards the membrane, are orientated to the right. (b) A skeleton diagram that indicates the position of the central layers of interacting side chains (numbered) in the neuronal SNARE core complex. Cα traces are shown in grey, the helical axes are highlighted by lines that are the same colour as the helices in part a, and the superhelical axis is highlighted by a black line. The ‘0’ layer is coloured red and all other layers are coloured black. (c) Overlays of individual layers, which are each shown contained in a shaded circle, from the neuronal SNARE core complex (grey) and the endosomal SNARE core complex (coloured). The endosomal SNARE core complex contains syntaxin-7 (Qa; red), VTI1b (Vps ten interacting-1b; Qb; light green), syntaxin-8 (Qc; dark green) and VAMP8 (R; blue). The three upper panels exemplify highly asymmetric layers that include the polar 0 layer and the –3 and +6 layers (the –3 and +6 layers contain conserved phenylalanines). The lower two panels show the hydrophobic layers that surround the 0 layer and are also highly conserved. Adapted from reference 12.
Membrane fusion by SNAREs and SM in five steps: (a) Membrane tethering. (b) Rab-regulated enrichment of fusion proteins and lipids in a microdomain. (c) Assembly of trans-SNARE with additional regulatory proteins such as SM and enrichment of lipids with small head groups (red head groups). (d) Hemifusion. (e) Completion of fusion and conversion of trans-SNARE complexes to post-fusion cis-SNARE complexes. Figure adapted from reference 14.
SNAREs are not uniformly distributed in plasmid membranes but rather clustered in membrane microdomains as directed by Rab5 (Figure 1.3). Rab5 regulates the enrichment of fusion proteins and lipids in a microdomain containing lipids such as sterols, phosphoinositides, or diacylglycerol. Enrichment of SNAREs by lipid microdomain formation and protein clustering provides high local concentration of fusogens and may result in more efficient fusion and vesicles readily fuse favorably at these microdomains. Rab5 regulation also results in a negative membrane curvature promoting hemifusion with lipids in the microdomain with small head groups. Following microdomain formation, trans-SNARE complexes are assembled with SM proteins (Sec1/Munc18) and can include other proteins or domains that bind to Ca\(^{2+}\), to lipids or to SNAREs, such as synaptotagmin and Munc13-1. SM proteins are composed of a conserved \~600–amino acid sequence that fold into an arch-shaped structure and can interact with SNAREs in different ways. Fusion of the apposing lipid membranes proceeds via a hemifusion step in which the outer monolayer are mixed while inner monolayer and its aqueous contents remain intact, to the opening of a fusion pore which allows both inner membranes and their contents to mix. Evidence suggests that SNARE complex formation drives membrane fusion by exertion of a mechanical force to their anchored membranes, which directly causes fusion. It is shown that the polypeptide membrane anchors of SNAREs can be replaced by passive lipid structures that span both leaflets. Current model implies that the linkers between the transmembrane domains and the SNARE motif is stiff, as it is a force transducer that passes the energy released by the zippering of SNARE complex formation. Fusion is blocked if a transmembrane domain is replaced by a more flexible lipid anchor, or if extra amino acids are inserted in
between the transmembrane domains and the SNARE motif.\textsuperscript{20,21} Post fusion, AAA+ (ATPases associated with various cellular activities) family protein NSF (N-ethylmaleimide-sensitive factor), along with SNAP (soluble NSF attachment proteins), couple the energy of ATP binding and hydrolysis to regenerate trans-SNARE complexes to cis-SNARE complexes. NSF does not interact with SNARE complexes on its own, it requires cofactors SNAPs (including $\alpha$-, $\beta$- and $\gamma$-SNAP) that bind to the SNARE complex. Both NSF and SNAPs are highly conserved and are likely to be involved in all SNARE complexes.\textsuperscript{22,23}

Although SNAREs represent the core of the fusion mechanism, the influence of the regulator proteins on SNAREs and the fusion reaction still need to be elucidated, as well as the mechanism of direct or indirect regulation by the protein kinases and phosphatases and other signaling proteins. As a result, clarifying the whole picture of SNARE mediated fusion reaction still remains challenging in many aspects.

1.1.4. Mitochondrial Fusion

Mitochondrial fusion is discussed separately here as an exception of intracellular fusion due to its distinct mechanism compared to that of the SNAREs. Because of the double-membrane organization, the mitochondrial fusion begins with the fusion of outer bilayer membrane, followed by the contact and fusion of inner bilayer membrane. Genetic studies have identified the main components involved in this process. Evidences demonstrate that proteins Fzo1 (in \textit{Drosophila melanogaster}) and Mfn1/Mfn2 (in mammals) are essential for mitochondrial fusion (Figure 1.4).\textsuperscript{24-26} They initiate the contact of outer membrane by the formation of anti-parallel coiled coils.\textsuperscript{27} Protein Mgm1/OPA1, a dynamin-related GTPase protein, which is localized in the inter-
membrane space and associated with inner membrane,\textsuperscript{28} has been found to play a direct role in mediating mitochondrial inner membrane fusion demonstrated by both in vitro and in vivo assays.\textsuperscript{29} Two possible pathways have been proposed on how Mgm1/OPA1 promotes the inner membrane fusion. The first possibility is that self-oligomerization of Mgm1/OPA1 acts like a SNARE complex. Another possibility is that Mgm1/OPA1 may promote the formation of highly curved region in the inner membrane.\textsuperscript{14} Another protein Ugo1 is an outer-membrane integral protein that seems to link Fzo1 to Mgm1 in the inner membrane.\textsuperscript{30}

\begin{center}
\textbf{Fig. 1.4.} Mitochondrial fusion. The fusion of mitochondria occurs on outer membrane first through the interaction of Fzo1/Mfn, and then inner membrane contact and fusion is regulated by Mgm1/OPA1. Ugo1 physically link Fzo1 and Mgm1. Adapted from reference 14.
\end{center}

1.1.5. Cell-Cell Fusion

Cell–cell fusion events are essential during mating, development and immune responses. Unlike other types of fusion that are mediated by a set of protein specifically, the molecular mechanisms of cell–cell fusion remain largely unknown. There is limited conservation from yeast to nematodes and insects to mammals, suggesting that these
might be unrelated mechanisms. Caenorhabditis elegans development is the best described cell-cell fusion process as they require anchor cell fusion failure-1 (AFF-1) and epithelial fusion failure-1 (EFF-1) proteins. They are both necessary for the fusion reaction and may oligomerize to form complexes in trans, though it still remains unclear how they mediate fusion in detail.

1.1.6. Fusion Mechanism and Simulation Studies

The fusion stalk mechanisms is thought to apply in many systems, (Figure 1.5) The first step is docking: the two membranes are brought into close proximity to counteract the electrostatic forces before the lipids of the proximal leaflets and interact. The second step is the formation of fusion stalk.

![Stalk model of membrane fusion](image)

**Fig. 1.5.** Stalk model of membrane fusion, adapted from reference 1.

In order to help understand the fusion pathway and analyze the fusogenic conditions, much effort has been spent on the modeling of the lipid bilayer fusion. Two major modeling strategies have been applied, namely, the continuum approach and the simulation approach.
**Fig. 1.6.** Pathway of lipid bilayer fusion through the formation of hemifusion stalk. (i) Pre-fusion contact. (ii) A point-like protrusion lowers the energy barrier for the two membranes to come into contact. (iii) A hemifusion stalk with one layer fused. (iv) Hemifusion diaphragm forms upon stalk expansion. (v) A fusion pore forms either directly from the hemifusion stalk or from the hemifusion diaphragm. Picture adapted from reference 79.

The continuum approach describes the membrane as macroscopic continuous film based on either elastic theory\(^{37-44}\) or self-consistent mean field theory.\(^{45-47}\) The approach uses the elastic model relying on the concept that the monolayer spontaneous curvature characterizes the intrinsic tendency of the monolayer to adopt a bent shape \(^{40}\) and the prediction of fusion intermediates comes from the bending, stretching and curvature parameters.\(^{48}\) The more sophisticated continuum approach uses self-consistent mean field theory, considering lipid bilayer as diblock copolymers composed of hydrophobic and hydrophilic homopolymers, and the water molecules as hydrophilic homopolymers.\(^{49-51}\) The continuum approach assumes that membrane fusion starts from a point-like membrane protrusion (Figure 1.6), followed by a hemifusion stalk and the stalk either decays directly into the fusion pore or undergoes the expansion to a round hemifusion diaphragm before the formation of the fusion pore.\(^{52-54}\)

The simulation approach uses direct-interaction forces to model membrane fusion, including Lennard-Jones interactions, screened Coulomb interactions, the angle
potentials, bond-mimicking interactions, and interactions of analogous types. The simulation approach depends on the model used to describe lipid bilayer and solvent. Most simulations confirmed the hemifusion stalk intermediate, although the simulation of the steps following that intermediate show different results using different models.55

The continuum models have been used to predict the factors that control the membrane fusion reaction, such as lipid composition, the thickness of the water layer, the membrane lateral tension and the curvature of the fusing membranes, which is also verified by simulations and experiments. In addition, two schemes of the generation of the membrane stress accumulated at the fusion site and released by stalk formation, including strongly curved membrane and intermembrane repulsion, have been demonstrated by the continuum approach and verified by the simulations. Estimates based on the continuum approach and verified by simulations will help elucidate the fusion pathway. Theoretical modeling of involvement of fusion proteins in the membrane fusion reaction hasn't been extensively studied.

1.1.7. Liposome Model Systems and Recent Mimicked Fusion Systems

The use of liposomes as models to study membrane fusion has advantages with regard to the following. First, synthetic membrane systems allow us to rigorously probe the role of each individual components in this complicated membrane fusion phenomena, by alternating lipid compositions, surface modifications, or other external conditions such as temperature, pH, or ionic strength. Second, varies fusion assays, such as lipid, content mixing or leakage assays are well developed and can be used to follow this process effectively. Third, liposomes are simple model systems that can provide us insight into finding the minimal requirements of membrane fusion.

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Many approaches have been taken under these guidelines. Researchers successfully mimicked membrane fusion by reconstituting synthetic v- and t-SNARE proteins into phospholipid membranes$^{10,62,63}$. Other systems less or unrelated to the native machinery were also found to fuse vesicles by using polyethylene glycol derivatives$^{64-66}$, amphiphilic peptides$^{67}$, or fusogenic lipid components$^{68,69}$. However, these approaches typically induce non-specific fusion, meaning poor selectivity between fusion partners. Molecular recognition provides a powerful tool to induce selective interaction at the interface of vesicular membranes that may result in fusion. One example is vesicle fusion induced by metal ion binding to amphiphilic ligands$^{70}$ (Figure 1.7), which happens only upon their coordination. Unfortunately, this process is not fully-characterized and the design of this system resulted in non-selective fusion between vesicles due to identical modifications of ligands on partner membranes.

![Fig 1.7. Fusion induced by metal-ligand coordination. M$^{2+}$ represents metal ions while Bn represents the ligand as displayed in pink arc on the left.](image)

More recently, researchers in this area developed various other approaches inducing selective membrane fusion. Boxer and coworkers$^{71,72}$ introduced a fusogenic system in which synthetic lipid–oligonucleotide conjugates inserted into lipid vesicles mediate fusion when one population of vesicles displays the 5’-coupled conjugate and the other
the 3'-coupled conjugate (Figure 1.8), resulting in both outer and inner leaflet mixing as well as limited (less than 3%) content mixing. Fusion regulated by DNA showed the importance of its orientation to the process because it is maximized using 5'- and 3'-coupled DNA as partners on opposite vesicles, rather than only 5'-coupled DNA. Dependence on sequence and average DNA number per vesicle were also observed. However, it is unclear, and somehow questionable that in these experiments, vesicles without DNA or presenting non-complementary sequences also appear to undergo some degree of lipid mixing or exchange (although no content mixing). A series of conjugates were constructed by adding 2–24 noncomplementary bases at the membrane-proximal ends of two complementary sequences to investigate the effect of membrane–membrane spacing on fusion. Increasing linker lengths generally resulted in reduced fusion probability and increased docking of complementary vesicles. The ability to control DNA sequence and the relative experimental simplicity provided by this system make it very attractive to probe fundamental questions of membrane fusion.

![Fig. 1.8](image)

**Fig. 1.8.** Schematic illustration of possible states in the DNA-mediated vesicle fusion reaction. Mixing of vesicles presenting 5'- and 3'-coupled DNA leads to docking, then to lipid mixing (shown here as a possible hemi-fusion intermediate), and last to content mixing (shown here as a possible result of full fusion of the vesicles). Adapted from reference 71.
Model systems using synthetic peptides that contain simplified versions of SNARE proteins\textsuperscript{73} (Figure 1.9) were also introduced recently. In their design of the lipilated peptides, LPE and LPK, the recognition domain is mimicked by two three-heptad repeat coiled-coil-forming peptides. These peptides are the shortest known coiled-coil pair to assemble specifically into a stable heterodimer (Kd=10\textsuperscript{-7} M). Formation of the LPE/LPK complex brings the two different liposomes close to fuse. A significant content mixing was observed in addition to lipid mixing. The similarity of this system compared to native SNAREs made this a very good minimal model for mimicking native fusion processes.

\textbf{Fig. 1.9.} Fusion system involving lipilated oligopeptides LPE and LPK. a) Space-filling model of the lipilated oligopeptides LPE and LPK, consisting of a DOPE tail linked through a PEG12 spacer to the coiled-coil-forming oligopeptides E and K. The amino acid sequence of E is G(EIAALEK)\textsubscript{3}-NH\textsubscript{2}, and that of K is (KIAALKE)\textsubscript{3}GW-NH\textsubscript{2}. b) The spontaneous incorporation of the DOPE tail in lipid bilayers results in liposomes decorated with either E or K peptides at the surface. When a liposome population carrying LPE (1) is mixed with a liposome population carrying LPK (2), coiled-coil formation (E/K) initiates liposome fusion (3). c) Comparison of the minimal model (left) with the SNARE-protein-based model (right). Adapted from reference 73.
However, native fusion processes such as exocytosis of synaptic vesicles are triggered on a millisecond time scale which turned out to be much more rapidly induced than synthetic systems. This discrepancy motivates us to delve deeper into understanding the native system. As an example, synaptic vesicles are exocytosed in a SNARE-dependent manner after pre-synaptic depolarization induces calcium ion influx. The Ca$^{2+}$ sensor required for fast fusion is synaptotagmin-1, which promotes SNARE-mediated fusion by inducing high positive curvature in target membrane in response to Ca$^{2+}$ binding$^{67}$. Other research shows that when the trans-membrane domains of SNAREs are replaced by covalently attached phospholipid anchors, fusion is prevented but the assembly of trans-SNARE complex docking vesicles is still allowed$^{74}$. Both examples suggest that not only recognition force is needed for fast fusion, but also membrane activation is playing a very essential role in this process.

**Fig. 1.10.** Schematic illustration of the vancomycin- D-Ala-D-Ala fusion system
Our first system\textsuperscript{75,76} employs vancomycin glycopeptide coupled to the antimicrobial peptide magainin II, and D-Ala-D-Ala-OH dipeptide coupled to a phospholipid derivative, as surface-bound fusogens (Figure 1.10). Here, magainin II peptide served as the role of membrane activation while recognition is driven by vancomycin and D-Ala-D-Ala-OH dipeptide. Fusion was characterized by dynamic light scattering and FRET experiments with lipid bound fluorophores, with regard to the effects of fusogen concentration, lipid composition, and membrane charge, etc.

![Diagram of membrane apposition and fusion](image)

**Fig.1.11.** (A) Schematic illustration of how membrane anchored lipids tri-functionalized with CA or M (symbolized as red and grey wedges) may direct lipid membrane apposition and fusion. (B) Possible models of inter-membrane hydrogen bonding, based on known CA/M assembly topologies: tape (left) and rosette (right).

Another system of ours involves replacement of vancomycin and D-Ala-D-Ala-OH by three cyanuric acid (CA) and melamine (M) (Figure 1.11),\textsuperscript{77,78} which drive surface recognition in water through hydrogen bonding and also lead to selective membrane
fusion and aggregation. The phenomena have been demonstrated in both liposomes and supported lipid bilayers (SLBs).

Liposomes are one of the ideal cellular cargo carriers because of their biocompatibility and ability to deliver lipophilic components or encapsulate hydrophilic materials inside the vesicles. To achieve this goal, liposomal cargo has to enter cells, and one of the most important ways is through fusion. Our research on membrane fusion and activation renders valuable knowledge in fusion kinetics, which should improve our understanding on using liposomes as delivery agents.

1.2. References for Chapter 1

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CHAPTER 2

SELECTIVE VESICLE FUSION VIA SMALL MOLECULE RECOGNITION BETWEEN KAA AND VANCOMYCIN

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2.1. Introduction

Modulation of lipid bilayer packing contributes to the activation of biomembrane functions\(^1\) and can initiate noncovalent membrane reactions such as lysis, pore-formation, and fusion.\(^2\) While many chemical agents can cause nonspecific lysis and fusion,\(^3\) native membrane chemistry is specific and regulated.\(^4\) The selectivity of membrane fusion is thought to arise from the coupling of membrane perturbation and surface-surface recognition (binding).\(^4,5\) Interestingly, both class I viral and endogenous secretory systems utilize protein coiled-coil recognition as an engine of fusion;\(^6\) indeed, these protein systems have been shown to fuse synthetic and biomembranes.\(^7\) Though fusion may also be induced using artificial membrane interactions,\(^8\) there has been no report of designed, controllable membrane fusion using well-defined small molecule recognition partners in apposing membranes. We report herein the induction of selective vesicle fusion with biological recognition motifs not natively associated with lipid bilayer fusion, thus broadening the scope of recognition-guided membrane activation.
2.2. Materials and Methods

2.2.1. Synthesis

Preparation of compound 9 Mono mesylation of pentaethylene glycol and a
subsequent reaction with sodium azide produced azide alcohol 6, which was oxidized with Jones reagent to carboxylic acid 7. Reduction of 7 followed by protection of the resulting primary amino group in 8 produced N-Boc-amino acid 9.

**Preparation of compound 10** N-Boc-amino acid 9 (351.2 mg, 1 mmol), TBTU (321.0 mg, 1 mmol) and DIEA (258 mg, 2 mmol) were dissolved in the DMF (5 mL) and stirred for 30 minutes. Methyl 4-[(aminoacetyl) amino] benzoate (1 mmol) DMF (5 mL) solution was added and stirred at room temperature for 3 hour, solvent was then removed under reduced pressure. The reaction residue was purified with flash column chromatography (methanol/methene chloride as eluting solvent) to give compound 10.

**Preparation of Compound 11** Compound 10 (120 mg, 0.22 mmol) was dissolved in water/methanol (v:v, 1:1, 3.0 mL) to which lithium hydroxide (4 mmol) was added and stirred for 30 minutes. The reaction mixture was acidified with HCl (1 M) and extracted with ethyl acetate. Organic layers were combined, dried with anhydrous sodium sulfate and solvent was removed to yield a white solid.

**Preparation of Compound 12** Compound 11 (121 mg, 0.23 mmol) was dissolved in methylene chloride (1 mL) and TFA (1 mL) was added. The reaction was monitored by TLC till its completion. Solvent was then removed and the remaining residue was dissolved in water to which saturated sodium bicarbonate aqueous solution (1.5 mL) was added, followed by N-methoxycarbonyl maleimide (47 mg, 0, 30 mmol). The reaction was stirred at room temperature for 1h, neutralized with HCl (1 M), extracted with ethyl acetate and dried with anhydrous sodium sulfate. Consequent solvent removal and flash column chromatography purification gave pure compound 12.
Preparation of compound 13, Compound 12 (27.8 mg, 0.06 mmol), TBTU (19.3 mg, 0.06 mmol) and DIEA (20 mg, 0.15 mmol) were dissolved in DMF (3 mL) and the reaction mixture was stirred at room temperature for 30 minutes after which POPE (43 mg, 0.06 mmol) was added in chloroform. The reaction was stirred overnight and solvent was removed after its completion. Resultant residue was purified by flash column chromatography yielding compound 13.

Vancomycin-Cys-Magainin Conjugate 4 Maleimide-functionalized magainin peptide synthesis was performed on Rink resin LS (0.2 mmol/g) using an Advanced Chemtech Apex Multiple Peptide Synthesizer, with diisopropyl carbodiimide (DIC) and N-hydroxybenzotriazole (HOBt) in N-methylpyrrolidinone (NMP) for couplings, and 50% piperidine in NMP for deprotection. The N-Terminus of the original 23 residue magainin 2 peptide was capped with maleimide-GGK(ABA), Side chain protecting groups were Lys(Boc), His(Trt), Ser(But), Glu(OBut), Asn(Trt). Cleavage of the peptide was effected with a (94:2:2:2) TFA, triisopropylsilane, thioanisole and water mixture for 2 hours, resin was removed by filtration and peptides were precipitated with ether (50 ml), centrifuged, and washed with ether (3×30 ml). Peptides were then dissolved in water and purified on a C18 semi-prep column (Higgins Analytical) eluting with a water/acetonitrile/TFA gradient, and lyophilized. Molecular weight was verified using a Bruker Esquire ESI or a Bruker Reflex III MALDI-TOF mass spectrometer. This peptide (10 mg) was then dissolved in water (1 ml) and to this was added phosphate buffer (1 ml, 0.5 mM, pH=7.0), mixed with 2 (5 mg, in 1 ml phosphate buffer, 2.5 mM, pH=7.0). Reaction was monitored by HPLC with an analytical C18 column (Higgins Analytical) by following UV absorbance at 230 nm, and the product was purified with a semi-prep C18 column.
(Higgins Analytical). Collected fractions were lyophilized to give 4. Molecular weight was verified with MALDI-TOF mass spectrometer. MALDI-TOF of 4, M.W. 4559 amu (Figure 2.2).

![MALDI-TOF spectrum of 4, M.W. 4559 amu.](image)

**Fig 2.2.** MALDI-TOF of 4, M.W. 4559 amu. (Mass 4433.744 and 4293.523 are due to the loss of sugars in the molecule)

Other compounds used in this chapter but not included here were prepared by Yumei Luo or Chun L. Yu.

**2.2.2. Fusion Assays**

**Liposome Preparation** Large unilamellar liposomes were prepared by the extrusion method. For a typical solution of labeled liposomes with Kaa-PE modification,
a lipidic film containing (95:1.5:1.5:2) molar ratio of egg PC, NBD-PE, Rh-DHPE and Kaa-PE was made by evaporation of the chloroform solution under nitrogen flow, and dried under reduced pressure overnight. The film was hydrated with Tris buffer (10 mM Tris, 100 mM NaCl, pH=7.4), and extruded 10 times both ways through a polycarbonate filter with 100 nm pore size. The final total lipid concentration was 2.5 mM.

**Fusion Assay (Dye Dilution)**  Fusion was followed at 25°C in 3.5 ml quartz cuvettes in a Perkin Elmer LS-50B fluorimeter (excitation 470 nm, slit width 10 nm/10 nm, cutoff filter 515 nm). For the 1:1 ratio (blank to labeled) fluorophore dilution assay, the “blank” liposomes (90:10 molar ratio of egg PC and POPG, 50 µl, 2.5 mM) were pre-incubated with 4 (93.6 µl, 13.36 mM, 1:100 molar ratio to the total lipid in “blank” liposomes) for 10 minutes, diluted with Tris buffer (1806.4 µl, 10 mM Tris, 100 mM NaCl, pH=7.4), and then added the fluorophore labeled liposomes with 3 modification (50 µl, 2.5 mM). Fluorescence emission from 515 nm to 620 nm was followed immediately after mixing in 2 minute intervals for 2-3 hours. Triton X-100 (100 µl, 20% (w/w)) was added to lyse the liposomes, giving the maximum values of NBD fluorescence.

**Reduction of NBD on the Outer Monolayer of Liposomes**  Liposomes with (96.5:1.5:2) molar ratio of egg PC, NBD-PE and 3 (5mM total lipid concentration) were reduced by mixing with an equal volume of sodium dithionite solution (100 mM Na₂S₂O₄,10 mM NaCl) in an ice bath for about 30 minutes until the NBD fluorescence was stable after a loss of ~50% intensity. The mixture was then filtered through an exclusion gel column (Sephadex G-25, Sigma-Aldrich) to remove the excess dithionite.
NBD fluorescence was measured before and after filtration to determine the final concentration of liposomes.

**Fusion Assay (Inner Monolayer Lipid Mixing)** Conditions were similar to the dye dilution assay. Liposomes containing 10% POPG labeled with Rh-PE (88.5:10:1.5, egg PC, POPG and Rh-PE, 50 µl, 2.5 mM) were incubated with 4 (93.6 µl, 13.36 mM) for 10 minutes, diluted with Tris buffer (1722.0 µl, 10 mM Tris, 100 mM NaCl, pH=7.4), and then NBD labeled, 3 modified liposomes were added (after reduction of the NBD fluorophore in the outer monolayer). Fluorescence was followed immediately after mixing for 2-3 hours with 2 minute intervals. Triton X-100 (100 µl, 20% (w/w)) was added to lyse the liposomes, giving the maximum values of NBD fluorescence.

**2.2.3. Dynamic Light Scattering**

Dynamic light scattering measurements were performed on a Brookhaven Instruments 200SM laser light scattering goniometer using a He-Ne 125 mW 633nm laser. The vesicle dispersions are placed into glass tubes. Single scans with 2 minute averaging time were performed on the sample with a 90 degree angle, using liposome solutions prepared under the same conditions as fluorescence measurements were done, in absence of fluorophores. Results were reproducible within 3 repetitions. All measurements were taken immediately after mixing.

**2.2.4. Mass Spectroscopy**

Electrospray mass spectroscopy was performed on a Bruker Esquire LC/MS plus ion trap spectrometer (Bruker-Daltonics, Boston, MA, USA) equipped with an electrospray ionization source was employed for mass detection. ESI was operated in positive ion mode to produce mainly protonated molecular mass ions (M+H)+1. MALDI-
Mass spectra were acquired on a Bruker Reflex III MALDI-TOF instrument. Peptide samples were dissolved in 0.1% v/v TFA in 50% v/v aqueous ACN. An aliquot (1 µL) of the peptide was spotted on a steel MALDI target plate (Bruker Daltonics) and α-cyanocinnamic acid (1 µl, supernatant of its saturated ACN/H$_2$O 1:1 v/v solution) was used as the MALDI matrix.

2.3. Results and Discussion

Our system employs vancomycin glycopeptide and D-Ala-D-Ala-OH dipeptide as surface-bound fusogens. Vancomycin binds to peptides that C-terminate in D-Ala-D-Ala with micromolar dissociation constants through the formation of a hydrogen-bonded complex with D-Ala-D-Ala. Membrane display of dipeptide and vancomycin derivatives (1 and 2) was accomplished by coupling to either phospholipid (3) or peptide (4) membrane anchors, respectively (Figure 2.3). Magainin 2, an antimicrobial peptide from frog skin, was chosen as an anchor for its ability to insert selectively into the hydrophobic matrix of negatively charged membranes and perturb lipid packing without vesicle fusion in the micromolar peptide concentration regime; there are possibly many other natural and synthetic peptides that could serve this role. We synthesized magainin itself and the vancomycin conjugate 4 (Figure 2.3) and then confirmed that both the synthetic peptide and conjugates bind preferentially to negatively charged membranes without significant change in vesicle size, as judged by dynamic light scattering (DLS, Figure 2.4). Indeed, initial screening by light scattering indicated that none of the individual compounds 1-4 caused appreciable change in size when incorporated at 1-5 mole percent, and therefore are not independently fusogenic (Figure 2.4).
However, mixing dilute liposome preparations displaying complementary binding partners 3 and 4 in trans (different membranes) resulted in a rapid increase in scattering (size), followed by a slower size decrease over 1-2 h to reach a stable size population on average larger than the initial mean diameter. Typically, 3 and 4 were incorporated into large unilamellar vesicles (LUVs) at 1% surface concentration: 3 was incorporated in neutral phosphatidyl choline (egg PC) lipids (3-LUVs); 4 was surface-bound with 10% phosphoglycerol in egg PC (POPG, negatively charged) lipids (4-LUVs). Notably, preincubation of 3-LUVs with free vancomycin suppressed changes in light scattering upon mixing with 4-LUVs, strongly supporting the notion that liposome aggregation is mediated by molecular recognition between vancomycin and D-Ala-D-Ala.

![Fig. 2.3. Structure of fusogens and intermediate compounds. Mercaptopropionamide Kaa 1 and vancomycin cysteine carboxamide 2 are reacted with maleimide-functionalized lipid and magainin anchors (red blue connection) to yield 3 and 4, respectively. The acetamidobenzamide moiety in 3 and 4 is a spectroscopic label.](image)
Fig 2.4. DLS of liposomes (A) with 2 mole % 3 in egg PC; (B) with 10 mole % POPG in egg PC; (C) contain 10 mole % POPG in eggPC, mixed with vesicles contain 2 mole % 3 in eggPC; (D) with 10 mole % POPG in eggPC, mixed with 1 mole% 4; (E) contain 2 mole % 3 in eggPC mixed with 1 mole % of 4; (F) Representative trace of size change as a function of mixing time on liposomes surface functionized with 3 and 4. Vesicles with 2 mole percent 3 in eggPC were reacted with liposomes composed of 10 mole % POPG in egg PC pretreated with 1 mole % 4; (G) Free vancomycin inhibits fusion. Representative traces of scattering change as a function of mixing time. Vesicles with 2 mole % 3 in eggPC, pretreated with 5 equivalents of free vancomycin were reacted with liposomes composed of 10 mole % POPG in egg PC pretreated with 1 mole % 4.

(Continued)
A membrane fluorophore dilution assay for fusion was used in which a lipid-bound FRET pair, N-(7-nitroben-2-oxa-1,3-diazol-4-yl)amine (NBD-PE) and rhodamine (Rh-DHPE), was incorporated at 1.5 mol % concentration each in cis (same membrane) with 3. Fusion of these vesicles with unlabeled LUVs displaying 4 should cause fluorophore dilution in the larger volume of the product membrane, consequently increasing donor (NBD) and decreasing acceptor (Rh) fluorescence, respectively. Indeed, vesicle mixing
resulted in this FRET fusion signature (Figure 2.5A); fusion began immediately upon mixing and slowed within 2h to a stable population, mirroring DLS results. As with DLS, 1, 4 and magainin itself were not fusogenic, and addition of free vancomycin blocked FRET change (Figure 2.5 A). Furthermore, dose dependent fusion was observed with the addition of an excess of blank liposomes bearing 4 to NBD/Rh/3 labeled liposomes. More than one full round of fusion was observed with a 1:9 excess within 20 minutes, comparable to fusion rates displayed by native fusion systems in LUVs (Figure 2.6).

Fig. 2.5. Representative traces of NBD fluorescence. (A) NBD-PE and Rh-HDPE dilution assay of fusion. Vesicles with 2/1.5/1.5 mol % 3/NBD/Rh in egg PC were reacted with liposomes composed of 10 mol % POPG in egg PC that were (O) pretreated with 1 mol % 4; (I) pretreated with 1 mol % magainin (without vancomycin coupled); and (4) 1 mol % 4 in the presence of 5 equiv free vancomycin to 3. (B) Inner monolayer mixing assay. NBD chemically quenched in outer monolayer of 2% 3-LUVs mixed with (I) 9 equiv, (4) 4 equiv, and (O) 1 equiv of LUVs bearing 1 mol % 4.

To distinguish this process from simple lipid mixing of the outer monolayers of each membrane, Rh/4 LUVs were reacted with NBD/3 LUVs in which NBD fluorophores on the outer monolayer were selectively destroyed by reduction with
sodium dithionite, which cannot effectively cross the membrane. FRET observed upon reaction of complementary recognition vesicles must result from the mixing of the inner monolayers that contain intact NBD, which is by definition membrane fusion. Gratifyingly, these experiments revealed a strong NBD/Rh FRET upon mixing of 3 and 4 derivatized liposomes, establishing this system as truly fusogenic (Figure 2.5B).

Fig. 2.6. (A) Rounds of fusion calibration curve. LUVs with 1:1 NBD/Rh prepared with increasing dilution to mimic FRET change caused by fusion. One round of fusion=dilution by a factor of 2. Less than one round of fusion represents a mixture of the liposomes at the original concentration and the one round dye diluted liposomes. (B) Rounds of fusion in dilution assay with (O) 9 equiv, (4) 4 equiv, and (1) 1 equiv donor (blank) to acceptor (NBD/Rh) LUVs.

Fusion requires a surface charge differential between donor and acceptor LUVs; as 2% 3 in egg PC LUVs contributes only 1% net negative surface charge, magainin and 4 remain bound to the LUVs with 10% POPG, in trans to 3. Liposome preparations in which both 3 and 4 containing LUVs had 10% PG lipids were non-or very weakly-fusogenic and nonaggregating as judged by light scattering and fluorescence
measurements. One plausible explanation for this is that flattening of the charge gradient between the two liposome populations allows the magainin vancomycin conjugate to equilibrate between the two membranes, leading to a decrease of membrane apposition and fusion rate.

These results indicate a fusion process in which surface binding initiates a highly aggregated state where fusion occurs rapidly and slows as the lipid binding partners increasingly occupy the same membrane: as membrane binding interactions in cis compete with interactions in trans, the number of binding partners available to catalyze membrane fusion decreases, as does the reaction rate and vesicular aggregation state. Interestingly, fusion rate is likely determined by the membrane location of the binding partner 4 and therefore the trans gradient of negatively charged lipids, which erodes as fusion proceeds. Further examination is underway to quantify the behavior of this system and evaluate its potential as a selective delivery vehicle. This initial study establishes the controllable and biomimetic ability of designed fusogens to activate specific membrane mergers in synthetic membranes via small molecule recognition; this has resonance with goals in targeted chemical delivery and nanoscale compartmentalized chemistry.

2.4. References for Chapter 2


CHAPTER 3

FUNCTIONAL DETERMINANTS OF A SYNTHETIC VESICLE FUSION SYSTEM

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3.1. Introduction

![Diagram of molecular recognition guided vesicle fusion.

Experimental and theoretical studies\textsuperscript{1, 2} concur that membrane fusion proceeds through at least 2 steps: membrane docking and actual fusion, resulting in the mixing of membrane lipids and membrane-bound contents (Figure 3.1). Fusion may occur upon close (1-2 nm)\textsuperscript{2} docking of target membranes, driven by the binding of surface groups. Docking “strains” the surfaces, allowing lipids from the two membranes to mix and
ultimately form a fusion pore connecting the two compartments. Insertion of a 
hydrophobic anchor into the lipid matrix can frustrate efficient lipid packing and activate 
the membrane towards noncovalent reactions such as lysis and fusion; these reactions are 
especially lipidic\textsuperscript{3} and are precisely controlled in Nature by molecular recognition events. 
Much of experimental data on selective membrane fusion has been gathered in studies of 
synaptic\textsuperscript{4} vesicle fusion machinery as well as in viral fusion machinery.\textsuperscript{5-7} Physical 
membrane deformation or insertion of a hydrophobic fusion peptide allows the formation 
of a high-energy intermediate non-bilayer lipid surface\textsuperscript{8} that fuses with its target 
membrane when drawn into apposition by surface binding. Native membrane recognition 
elements are proteins, which in class I viral fusion\textsuperscript{5} and synaptic vesicle fusion are coiled-
coils. Helical bundle formation draws the membranes into apposition; this binding is 
thought to locally dehydrate and mechanically deform the membrane, lowering the 
activation energy for lipid mixing and fusion.\textsuperscript{1} Notably, enveloped viruses such as HIV\textsuperscript{6} 
and influenza\textsuperscript{7} similarly employ coiled-coil recognition to guide fusion with the host membrane. That all known native membrane fusion is driven by protein recognition (and 
often with coiled-coils), raises the question of whether recognition strategies between 
small molecules would also be fusion-competent. Such a minimal fusogenic molecular 
system would be useful to determine the fundamental requirements for membrane fusion 
catalysis. A simple synthetic model system of specific fusion via molecular recognition 
would allow physical organic methods to be applied to rigorously probe the scope and 
limitations of controlled lipid membrane fusion. All known molecular recognition 
systems that induce selective fusion are derived from native fusogenic proteins that must 
be expressed; further, their fusogenic behavior is complex and modulated by other
species in vivo.\textsuperscript{9} Thus, detailed study of membrane fusion phenomena using native fusogenic systems as probes is complicated by limited control over chemical content and the participation of multiple proteins in the native system. Though there have been reports of small molecule fusogenic systems,\textsuperscript{10,11} these systems lack controlled valency, defined partner selection and are not thoroughly characterized. We have previously reported a well-defined chemical model system which has allowed us to examine the molecular determinants of membrane fusion.\textsuperscript{12} We report here our detailed examination of the parameters that define membrane fusion behavior. Our designed fusion system incorporates the two functions of recognition and disruption as compactly as possible (Figure 3.2).

Fig. 3.2. Selective vesicle fusion guided by vancomycin/ D-Ala-D-Ala recognition.

We chose vancomycin glycopeptide and D-Ala-D-Ala dipeptide as a recognition pair to guide fusion. Vancomycin is an antibiotic of last resort that blocks the transpeptidation step of peptidoglycan synthesis by binding to D-Ala-D-Ala dipeptide on
lipid II, a key biosynthetic intermediate of the peptidoglycan cell wall of bacteria. Recognition occurs via the formation of 5 hydrogen bonds between the two peptide backbones and the free C-terminus of the dipeptide (Figure 3.3). Due to its therapeutic importance, this micromolar dissociation binding event is very well studied. Strikingly, when the vancomycin interaction is made trivalent by connection to a rigid scaffold, the dissociation constant decreases impressively to $10^{-17}$ M. Though less structured, membrane ligand presentation also exhibits increased binding avidity, which may overcome surface repulsion energies and result in membrane apposition. We therefore judged the vancomycin / D-Ala-D-Ala recognition pair to be an ideal starting point for exploration of small-molecule triggered fusion: the importance of vancomycin has led to a large body of literature on how it interacts with D-Ala-D-Ala as well as synthetic methodology for modification of the commercially available drug.

![Fig. 3.3. Vancomycin (top) binds to Lys-D-Ala-D-Ala (bottom) via 5 hydrogen bonds.](image-url)
Fig. 3.4. Synthetic fusogens 1 (vancomycin-magainin conjugate) and 2 (Lys-D-Ala-D-Ala phospholipid).

Frog skin-derived antimicrobial peptide magainin II,\textsuperscript{16, 17} was used to anchor vancomycin to the membrane for reasons similar to our choice of recognition pair: magainin is a very well-studied peptide whose membrane binding mode is known, and it is also known to destabilize (perturb) membranes in a concentration dependent manner. Lipid packing and vesicle diameter determines membrane curvature and overall strain,\textsuperscript{18} thus, membranes frustrated by hydrophobic shape mismatch are activated for noncovalent reactions such as fusion since strained non-lamellar structures must be formed from the low curvature bilayer state upon fusion. While phospholipases can increase the rate of fusion by producing single chain lipids that are hydrophobically mismatched with 2 chain lipids,\textsuperscript{19} noncovalent modification by peptide insertion can activate the bilayer for reaction in much the same way. Shallow insertion of a
hydrophobic anchor such as a peptide into the top monolayer of a bilayer (Figure 3.4) can create negative curvature,$^{20-22}$ which destabilizes the lamellar phase. Many antimicrobial (AMPs)$^{16,21,23}$ and viral fusion peptides$^{24}$ insert in this way and activate permeation and fusion by lowering the energy barrier to non-bilayer lipid phases. Permeation and lysis are dose-dependent, and in low concentration AMPs may bind to membranes without lysis or fusion and minimal permeability increases. Thus, natural product AMPs and viral fusion peptides are possible perturbative components of designed fusogenic systems if coupled to molecular recognition motifs. Magainin II binds selectively into the hydrophobic matrix of negatively charged membranes and perturbs lipid packing without vesicle fusion in the micromolar peptide concentration regime; there are possibly many other natural and synthetic peptides that could serve this role.$^{25}$ Thus, we have previously prepared synthetic fusogens 1 and 2 (Figure 3.4) and verified that they can indeed selectively induce fusion of vesicular membranes via specific molecular recognition of surface-displayed vancomycin and Kaa peptides. We present herein detailed study of the parameters affecting membrane fusion in this system.

3.2. Material and Methods

**General methods and instrumentation.** All synthetic reactions were carried out at room temperature. All fluorescence, CD, and ITC experiments were thermostatted at 25 °C. Stock solutions of fusogens 1 and 2 were prepared and concentrations determined using acetamidobenzamide absorbance ($\varepsilon_{270}=18,000$). Lipid fusogen 2 was dispersed in lipid films at 1.0-2.0 mole percent while peptide fusogen 2 was added to preformed LUVs at 1 mole percent concentration. Lipid film hydration with was used to prepare a polydisperse population of multilamellar vesicles, which were extruded through a 100 nm
polycarbonate filter to obtain large unilamellar vesicles\textsuperscript{36} (LUVs) with diameters ranging from 120 to 140 nm as judged by dynamic light scattering measurements. LUVs were prepared using mixtures of neutral egg phosphocholine lipids (egg PC) and negatively charged phosphoglycerol (POPG) lipids. Fluorescence measurements were performed in a Perkin-Elmer LS-50B using a 3 ml cuvette or in 96-well plate format with a Spectramax M5 plate reader (Molecular Devices). Circular dichroism measurements were made with an Aviv CD spectrometer. Titration calorimetry experiments were run using a VP-ITC Microcalorimeter. Peptides were synthesized on an Advanced Chemtech (Apex 396) automated peptide synthesizer using standard Fmoc chemistry and purified to homogeneity on reverse phase HPLC. Purified peptides and vancomycin derivatives were identified by ESI-MS or MALDI-MS, as were purified lipid derivatives, prepared as previously described.

Materials. Fluorescent lipids NBD-PE and Rh-DHPE were purchased from Invitrogen and used as provided by the manufacturer. Phospholipids POPG, DPPC and egg PC were purchased from Avanti Polar Lipids. Amino acid derivatives were purchased from Advanced Chemtech and EMD Biosciences, vancomycin hydrochloride and other fine chemicals were purchased from Aldrich and used as provided.

Addition of Kaa thiol to POPE lipid anchor. S-Trityl and N-Boc protected peptide (TrtS- (CH\textsubscript{2})\textsubscript{2}CO\textsubscript{2}-Lys(Boc)--OH, 28 mg, 38 \(\mu\)mole) dissolved in a minimum amount of TFA (200 \(\mu\)L), yielding a bright yellow solution which was diluted with 2 ml ethyl ether after 2 minutes. The suspension was centrifuged to obtain a white pellet. The supernatant was removed and the pellet which was resuspended in fresh ether and spun down twice more to remove TFA. The white precipitate was dissolved in degassed
methanol and added directly to a methanol solution of POPE anchor 6 (30 mg, 25 µmol) with 6 equivalents DIEA under argon. Reaction was followed by HPLC and purified to homogeneity on a C4 column with a gradient of 70% solvent B to 100% solvent B in solvent A over 40 minutes (solvent A=4.99% methanol, 95% H2O, 0.1% formic acid; solvent B=4.99% H2O, 45% methanol, 50% isopropanol, 0.1% formic acid). Product mass was confirmed by electrospray mass spectroscopy (calculated: 1539 amu, found: 1540.2).

Membrane binding experiments. All peptide lipid titrations were performed using 10 mM Tris buffer, pH 7.4, 100 mM NaCl. Changes in signal upon interaction with lipid (ellipticity, heat) were normalized by the maximum change in signal for each experiment after background subtraction. The resulting normalized fractional change in signal at each step of the titration was taken as fractional binding. Each addition of titrant was followed by equilibration time; for ITC this was taken as the time required for heat flow to return to baseline and for CD equilibration time was 3 minutes.

3.3. Results and Discussion

3.3.1. Synthesis of Lys-D-Ala-D-Ala-phospholipid derivatives

The binding partner to 1 was similarly prepared by coupling a synthetic mercaptopropionamide capped tripeptide sequence from the lipid II peptide, Lys-D-Ala-D-Ala (Kaa) to a palmitoyl-oleoyl phosphatidyl ethanolamine (POPE) lipid derivative.12 This POPE lipid anchor was functionalized with a PEG linker26, 29 bearing a terminal maleimide (Figure 3.5). Both the magainin and POPE anchors bear an acetamidobenzamide (ABA) moiety as a UV label (ε270=18,000 M⁻¹cm⁻¹) to determine concentration. While Lehn and co-workers have shown that long PEG linkers are
necessary used to induce fusion in LUVs upon metal complexation with tethered
ligands, we have used relatively short linkers to minimize the entropic cost of surface
binding and to avoid possible complications from PEG-induced membrane activity.

With these maleimide-derivatized anchors in hand, preparation of peptide and lipid
fusogens 1 and 2 was straightforward (Figure 3.6). Mixing vancomycin-cysteinamide
with the magainin anchor in water in a 1:1 ratio resulted in clean formation of the adduct
upon mixing, which was purified on HPLC. Similarly, phospholipid derivative was
reacted with the Kaa-mercaptopropionate in methanol with diisopropylethylamine to
yield fusogen 2, which was purified by reverse phase HPLC.

**Fig. 3.5.** Preparation of linkers for phospholipid fusogen 2. (a) MsCl, Et$_3$N, THF; (b)
NaN$_3$, MeOH; (c) CrO$_3$, acetone; (d) HS(CH$_2$)$_2$SH, Et$_3$N, MeOH; (e) Boc$_2$O, KOH, THF,
H$_2$O; (f) TBTU, DIEA, DMF; (g) LiOH, MeOH, H$_2$O; (h) TFA; (i) N-methoxy-
carbonylmaleimide, Et$_3$N.
3.3.3. Magainin-vancomycin conjugate binding to large unilamellar vesicles (LUVs).

The binding of magainin to negatively charged membranes is well established, as is its inability to bind to neutral membranes. Our magainin-vancomycin conjugate is designed to bind to both negatively charged membranes and any membrane bearing Lys-D-Ala-D-Ala (Kaa) lipid fusogen 2. To better understand the role of the peptide anchor in fusion, we set out to determine the binding constants of our magainin-vancomycin fusogen 1 to both negatively charged and Kaa displaying vesicular membranes. Fusogen 1 is random-coil (unfolded) in aqueous solution, but rapidly folds into an α-helix as judged by circular dichroism (CD) upon interaction with LUVs with 10% and 20% negative charge from palmitoyl-oleoyl phosphoglycerol (POPG) lipids. While 1 did not fold with neutral phosphocholine (PC) LUVs, POPG LUVs induced a lipid dependent coil-helix transition. There is clearly a strong electrostatic component for surface binding as the CD signal increases more intensely and rapidly with increasing POPG content in the LUV titration. The lipid-dependence of helicity was analyzed using a surface-partitioning model, in which the partitioning constant $K_p$ is defined as:

Fig. 3.6. Fragment coupling to produce fusogens 1 and 2.
\[ K_p = \frac{C_b / C_l}{C_{p,\text{free}}} \]

wherein in \( C_b \), \( C_l \) and \( C_{p,\text{free}} \) are concentrations of surface-bound peptide, total lipid and free peptide in solution. Thus, when surface concentration \( X_b \ (C_b/ C_l) \) is plotted as a function of \( C_{p,\text{free}} \), the slope yields the partitioning constant \( K_p \):

\[ X_b = K_p \times C_{p,\text{free}} \]

This model assumes that the partitioning constant does not change with bound peptide concentration, an assumption which breaks down when binding is driven by electrostatic interactions or specific lipid binding.\(^{30,32} \) In these latter cases, the propensity for membrane partitioning decreases the more peptide is bound, due to either decreased surface potential from the positively charged peptide, or as a result of saturation of the surface binding sites. Thus, simple partitioning yields a linear \( X_b \) vs. \( C_{p,\text{free}} \) plot, while situations with both hydrophobic partitioning and a secondary interaction (electrostatics or specific recognition) exhibit non-linear saturation behavior. In the non-linear plot, the apparent partitioning constant may be calculated at each peptide concentration. We performed this analysis for the binding of magainin-vancomycin to vesicles bearing a 10\% and 20\% negative charge as well as neutral vesicles bearing 2\% D-Ala-D-Ala lipid, using CD and isothermal titration calorimetry (ITC) to follow binding, respectively (Figure 3.7).
The 10% POPG membrane exhibits a roughly linear fractional binding plot, consistent with weak electrostatic binding, with an apparent $K_p$ (slope) of 1300 M$^{-1}$ (data not shown). As charge content increases to 20% POPG, the $X_b$ plot becomes significantly
non-linear, indicative of a partitioning constant that decreases as more cationic peptide is bound. Notably, binding to the 2% Kaa membrane saturates at a surface concentration $X_b$ of ~0.01 (1%), indicating two points: 1) the peptide fusogen 1 can only bind the Kaa on the *outer* monolayer of the vesicle, which has roughly half the total Kaa, and 2) binding is completely driven by specific Kaa lipid recognition by 1. The 20% POPG surface has a higher binding capacity for fusogen 1, saturating at 3% surface concentration. Furthermore, fractional binding to the POPG surface is consistently higher than the Kaa surface at the same equilibrium free peptide (fusogen 1) concentration. Though scatter noise from high lipid concentration in the CD titration prevents exploration of lower peptide-lipid ratios, the trend clearly indicates that partitioning into the 20% POPG membrane is favored over binding to the 1% Kaa membrane. For example, when the equilibrium concentration of peptide fusogen 1 is 1 $\mu$M in solution, the apparent partitioning constant the membrane is 7000 M$^{-1}$ as compared to 18,000 M$^{-1}$ for the POPG membrane. The difference in partitioning constants should widen further at lower free peptide concentrations. It is clear from this analysis that under the conditions of the fusion reaction, there is effectively no free peptide in solution; all is membrane-bound, and mostly on the POPG membrane. A 1% loading of fusogen 1 correlates to a peptide-lipid ratio of 0.01 on 20% POPG LUVs, which is completely bound ($X_b=0.01$), whereas the same loading on a 1% Kaa membrane is only 70% bound ($X_b=0.007$) (Figure 3.7). Importantly, although electrostatics drives peptide fusogen 1 partitioning more so than specific lipid recognition, the partitioning constants are still within the same order of magnitude, meaning that fusogenic peptide has significant affinity to both POPG and Kaa
membranes; this is in line with expectations for a peptide that acts to drive apposition and fusion of these two surfaces.

### 3.3.4. Fusion Is Catalytic in Peptide Fusogen 1

The observation that charge content greatly affects fusogen binding suggested that fusion rate should depend on surface charge differential between the two fusing LUVs. This is likely the reason why fusion slows and stops; the charge gradient between the two liposome populations erodes as fusion proceeds, leading to product inhibition and eventual halting of fusion as fusogen 1 binds preferentially to the product (fused) membrane which has both negative charge and Kaa lipid, forming a “cis” complex. Thus, we reasoned that a fusion system catalytic in 1 would be possible. One round of fusion between neutral and 10% POPG-LUVs results in product liposomes with 5% POPG and unreacted 10% POPG LUVs. Addition of 20% POPG LUVs should competitively bind all 1 in the system as the partitioning constant is 1-2 orders of magnitude higher than to 10% POPG, permitting the formation of new “trans” complexes and a new round of fusion.

![Fig. 3.8. Charge gradients render fusion catalytic in peptide fusogen 1.](image)
FRET dilution experiments\textsuperscript{33} confirm that this indeed happens (Figure 3.8). Using the same fusion assay as in previous studies,\textsuperscript{12} we incorporated NBD and rhodamine functionalized lipids into LUVs with Kaa lipid 2 and treated this suspension with POPG LUVs and peptide fusogen 1; increase in NBD (donor) fluorescence was taken as a reporter of loss of FRET, membrane dilution and therefore fusion. We allowed a fusion reaction with a 10\% negative charge differential come to equilibrium, then charged the system with 1 equivalent of 20\% POPG LUVs, \textit{without additional fusogen 1}. This resulted in a second burst of fluorescence change, suggesting that new fusion active LUVs may be formed if the charge gradient is sufficient. The system showed a response to multiple injections of naked 20\% POPG LUVs, with diminishing response as expected with the eroding charge gradient between product and unreacted LUVs. Thus, sequential rounds of fusion may be accomplished with this strategy; these characteristics indicate an artificial fusogenic system whose behavior is not only robust, but predictable (Figure 3.9).

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{dye_dilution_fusion_assay.png}
\caption{Dye dilution fusion assay. 4:1 donor (1/10\% POPG) to acceptor (2\% 2 in ePC) LUVs. 1 equivalent LUVs (20\% POPG) added at (a) 168 minutes, (b) 289 minutes and (c) 409 minutes.}
\end{figure}
3.3.5. Effects of altering charge content and ligand concentration on fusion rate

To determine the effects of varying the relative affinity of peptide fusogen to either reacting membrane, fusion rate was measured as a function of charge content in the membrane as well as surface concentration of fusogen Kaa lipid. Interestingly, analysis of initial rates indicates that there is a minimum concentration of ~0.5 % lipid required before fusion will occur; after this “turn on” concentration, fusion rate increases steadily and ultimately levels off at higher Kaa lipid concentration (Figure 3.10). The dependence on surface fusogen concentration suggests there must be a minimum level of fusogen partitioning into the Kaa membrane before the membranes are activated enough to fuse. A similar experiment was undertaken by varying charge content in the apposing membrane. In this case, 10% POPG LUVs fused slower than 20% POPG, but above 20% negative charge, fusion rates decreased sharply with a significant change in rate profile (Figure 3.10). We speculate that more negatively charged membranes bind the magainin anchor of the peptide fusogen too tightly to allow the pendant glycopeptide to interact with its Kaa binding partner. Unlike vancomycin/Kaa lipid binding, magainin-bilayer interactions involve the entire length of the peptide anchor, and tightening this binding by increased electrostatic interactions may decrease steric accessibility to the Kaa reactant membrane. This does not appear to be an issue when Kaa lipid concentration is increased, possibly because the magainin-vancomycin conjugate 1 is only anchored in the membrane at a single point by glycopeptide-lipid recognition, with the magainin anchor likely partitioning weakly in the neutral PC membrane. These differences in the way fusogen 1 anchors to the two reacting membranes may have important functional consequences, as discussed below.
Fig. 3.10. Fusion rates are represented by relative change in NBD fluorescence. (A) Fusion as a function of Kaa lipid fusogen 2 mole percent in membrane. (B) Initial rates for fusion as function of Kaa surface concentration. (C) Fusion as a function of percentage of POPG in membrane.
3.3.6. Peptide-Membrane binding and Membrane Activation

Vancomycin- binding facilitates insertion of the magainin anchor into a neutral egg PC membrane, as judged by the Kaa LUV dependent CD spectrum, which shows peptide helicity increasing with lipid concentration (Figure 3.11). Broader implications may be drawn for peptide membrane insertion mechanism: it appears that it is sufficient to bring the peptide close to the membrane and insertion becomes spontaneous, perhaps once the lipid headgroup hydration layer is passed. The sequence of many AMPs favors insertion in the lipid matrix though not all of these peptides (such as magainin) insert spontaneously, requiring assistance from electrostatic interactions. This suggests that the major impediment to insertion may be a kinetic barrier, represented by the hydration layer; water molecules hydrating the lipid headgroups must be pushed aside to allow peptide approach and subsequent insertion. Lipid recognition could provide the binding energy to bridge the hydration layer, after which partitioning is spontaneous. This apparently may be accomplished by non-specific favorable electrostatics or with specific molecular recognition. These two different insertion pathways have strikingly different effects on membrane function.

We studied contents release upon fusogen 1 binding with vesicles that encapsulated dye at self-quenching concentration; we noted that peptide fusogen 1 induces rapid dequenching (release) of contents from vesicles with 1% Kaa lipid fusogen 2, whereas 1 binding to 20% POPG LUVs under similar conditions elicits no detectable contents release. This suggests that peptide insertion driven by specific lipid headgroup recognition is much more disruptive than insertion of an amphipathic peptide into the membrane. Clearly, the physical parameters of insertion are different. While
electrostatically driven binding results in roughly uniform insertion of the hydrophobic face of helical magainin, specific binding interaction with a lipid headgroup dictates that the binding terminus must remain out of the membrane while the other end is inserted (Figure 3.11).

Fig.3.1. (A) CD monitoring of lipid to magainin-vancomycin (MV) titration. 2 mole % Kaa-lipid in eggPC liposomes are titrated into the magainin-vancomycin conjugate. (●) lipid:peptide mole ratio = 0; (▲) lipid:peptide mole ratio = 30; (○) lipid:peptide mole ratios in between 0 and 30, all traces decrease at 222 nm with ratio=30 the lowest. (B) Dye release from liposomes upon treatment with MV. Green trace = 2% Kaa-lipid in eggPC, lower traces = 20% POPG in eggPC and 100% eggPC. (C) Model for how lipid headgroup binding can alter peptide insertion angle and result in more disruptive insertion.

(Continued)
The latter mode would involve only partial insertion which would make it more difficult it is for the lipid matrix to compensate for lipid packing defects. This is similar in concept to the greater membrane activity of obliquely inserting peptides versus transmembrane inserted peptides. Transmembrane insertion merely compresses lipid packing, whereas oblique or partial insertion frustrates packing in the outer lipid monolayer only. This study supports the notion that specific lipid recognition\textsuperscript{38} can
dramatically alter the functional and physical consequences of peptide-membrane insertion by controlling peptide insertion; presumably, insertion angle and depth are important parameters. This finding lays the groundwork for future designs of membrane-activators, including selective fusogenic and pore-forming systems.

3.3.7. Effect of lipid composition on contents leakage, domain formation and fusion rate

One function of cholesterol in biomembranes is to fill in packing defects and stabilize unsaturated lipid membranes.\(^{39}\) We hypothesized that peptide insertion was causing lipid matrix packing defects that resulted in permeability increases and thus we incorporated cholesterol in reacting membranes in an effort to decrease leakage during fusion. Using the Cbf release assay as above, we discovered that cholesterol additives slowed leakage, but also slowed the fusion reaction. The latter does make sense, as the more rigid the membrane, the less reactive it should be, though the reason why cholesterol does not significantly decrease leakage is unclear. However, when dipalmitoyl phosphatidyl choline (DPPC) was added to the egg PC membrane, leakage decreased and fusion rates increased, in conjunction with increasing DPPC content (Figure 3.12). We interpret these results in the following way: the increased DPPC content causes the membrane to rigidify and thus decrease leakage, while increased fusion rate reflects domain formation in the membrane. It has been reported that mixtures of saturated and unsaturated lipids such as DPPC and POPC or POPG will phase separate,\(^{40}\) resulting in separate fluid and gel phase membrane domains. Cholesterol has the opposite effect, causing a mixing of domains that results in one liquid crystalline domain that is both strong and fluid, which is critical for biomembranes.\(^{39}\)
These biophysical properties of DPPC and cholesterol containing membranes may be the underlying cause of the observed chemistry.

**Fig. 3.12.** Fusogenic LUVs are mixed at t=0. Donor LUVs preincubated with magainin-vancomycin conjugate and all have 20% POPG and vary in ePC/DPPC ratio: ●=80% ePC; ○=60% ePC, 20% DPPC; □=40% ePC, 40% DPPC; △=20% ePC, 60% DPPC. (A) Fusion rate: acceptor LUVs composed of 2% Kaa lipid, 1.5 % NBD-PE, 1.5% Rh-lipid, 40% DPPC in ePC. (B) Dye release: acceptor LUVs composed of ePC/2% Kaa encapsulating Cbf at self-quenching concentration.
Domain formation in a POPG/DPPC/eggPC membrane would segregate the fluid (POPG, egg PC) from the gel (DPPC), and also segregates the negative charge (POPG) from the gel. Therefore, binding of the magainin-vancomycin fusogen would occur on the negatively charged fluid phase island only (Figure 3.13). The POPG domain would have higher charge density and the membrane as a whole would have peptide binding directed to a surface subdomain instead of being uniformly distributed. This would increase both the density of ligands and degree of activation, thereby increasing the avidity and productivity of initial membrane apposition, resulting in faster fusion.

**Fig. 3.13.** Possible domain interaction leading to enhanced membrane activity.

### 3.4. Conclusions

We have presented herein studies which identify the aspects of our designed membrane fusion system that are critical for reactivity. These findings indicate that membrane activation for fusion depends strongly on the way the fusogen anchors into the lipid matrix. “Single-point” attachment via specific lipid recognition by a peptide results in much more disruptive binding than “multi-point,” as when the entire helical face is buried by electrostatically driven binding. This suggests that insertion depth and angle of peptide helices can determine membrane activity, and further indicates that specific lipid recognition may generally enhance the membrane activity of all antimicrobial peptides.
The presence of gel-phase lipids was found to significantly increase membrane fusion rate and decrease leakage, which provides support for the notion that membrane subdomains formed by lipid mismatch may serve to cluster lipids and fusogens in the bilayer, enhancing binding and function. While the functional role of such lipid rafts in biology remains contentious, their physical existence in synthetic membranes is well established, and this study suggests how lipid subdomains may play a functional role in a synthetic fusion system. Examination of this synthetic model system has yielded insights into both fusion and permeation processes which are general for the lipidic phase of membrane function, and thus have relevance these same processes in biological membranes.

3.5. References for Chapter 3


CHAPTER 4

LIPID MEMBRANE ADHESION AND FUSION DRIVEN BY DESIGNED, MINIMALLY MULTIVALENT HYDROGEN-BONDING LIPIDS

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4.1. Introduction

Selective aqueous phase hydrogen bond-driven molecular recognition faces the challenge of solvent competition. While there are many solutions to this problem in Nature, there are few non-native designed systems known. Our group has previously reported the aqueous-phase studies on cyanurate (CA) and melamine (M) phospholipid derivatives that drive membrane chemistry via hydrogen-bonding interactions between the CA and M headgroups. There have been numerous studies on the CA-M system; though the parent compounds readily co-crystallize in a hydrogen-bonding network in water and derivatives are well-known to assemble in low dielectric solvents or when hydrophobically buried, derivative assembly generally does not occur in hydrogen-bonding solvents. Indeed, without the phospholipid module, monoderivatived cyanurate (CA) and melamine (M) neither assemble nor inhibit interactions between the lipids in water. Surface multivalency of an assembled membrane provides binding avidity that greatly enhances the hydrogen-bonding interaction at the lipid-water interface. The requisite density of CA/M modules for
recognition was indicated by diminished interaction at CA-PE/M-PE concentrations of less than 70% in fluid phase (ePC) membranes. Notably, when CA-PE and M-PE were diluted in membranes with gel-phase phospholipids (DPPC), 30% of CA/M lipid was sufficient for vesicle binding, presumably due to clustering of CA/M headgroups upon phase separation of CA/M-PE lipids from the gel-phase lipids. These findings prompted our investigation of the minimal valency required for detectable lipid-lipid binding. This exploration was guided by the notion that each lipid can only interact with its surrounding neighbors at any given moment; thus, recognition must be facilitated by nearest neighbors. Separation of hydrogen-bonding (HB) groups by just one lipid (50 mole percent concentration in ePC) abrogates lipid-lipid binding, implying that clustering of HB groups is essential. We synthesized lipids in which three CA or M groups were forced into proximity by covalent attachment to the same phospholipid and found that this minimal design imparts robust molecular recognition at the lipid-water interface.

4.2. Materials and Methods

Chemicals. Fluorescent lipids (NBD C6-HPC, NBD-PE, Rh-DHPE, Oregon Green 488-DHPE and Texas Red-DHPE) were purchased from Invitrogen. Phospholipids POPG and ePC were purchased from Avanti. Starting materials for synthetic headgroups were purchased from Sigma-Aldrich. All chemicals were used as provided by the manufacturer.

Fusion Assay of Lipid Mixing. Fusion was followed at 25 °C on a Perkin Elmer LS-50B fluorimeter as described earlier. Total lipid concentrations were 0.2 mM, with the two LUV populations at 1:1 molar ratio. Maximum NBD fluorescence was obtained
by liposomes lysis with 100 μl of n-dodecyl-β-D-maltoside solution (5 wt% in water) and corrected for dilution.

**Supported Lipid Bilayer Formation.** Glass slides or cover slips were boiled in detergent solution (Linbro 7X Lab Glass Cleaner, ICN Pharmaceuticals) for 20 minutes, followed by extensive rinsing, and then baked at 450 °C for 4 hours. Supported lipid bilayers were formed by exposing glass slides or cover slips to a sample of desired liposomes (lipid concentration ≥0.25 mM) for five minutes, followed by rinsing with excess DPBS buffer, keeping lipid bilayer hydrated at all times.

**Microcontact Printing and Converging Channel Formation.** Formation of fibronectin grids was performed according to published procedures with minor modifications: Poly(dimethylsiloxane) (PDMS) stamps for microcontact printing were formed by curing Sylgard 184 (Dow Corning) on a silicon master (a generous loan from Steven G. Boxer, Stanford University) with patterned photo-resist. Stamps (1x1 cm) were cleaned in a plasma cleaner (Harrick Plasma) for 20 seconds at the highest power setting with a slight leak to room air. The patterned side was soaked with 50-100 μl of 100 μg/ml fibronectin (Sigma) in DPBS buffer for 20 minutes, then dried under a stream of nitrogen. Stamping was done by inverting the stamp and placing it on a cleaned glass slide or cover slip with a 40g weight on top for 20 minutes. After that the glass slide was rinsed with buffer, water, and then dried under a stream of nitrogen. For FRAP experiments, a chamber gasket (Invitrogen) was attached on top of the patterned glass surface; for experiments involving the converging channels, two layers of PDMS elastomer were cut using a template and layered to produce three walls of the converging channel. Channel was assembled on top of the patterned glass, which comprised the
fourth wall of the channel. The silicon master for the 50 μm grid was a generous loan from Steven G. Boxer (Stanford University).

**Microscopy Imaging.** Epifluorescence microscopy for the samples was performed on a Zeiss Axio Observer A1 inverted microscope equipped with a mercury light source, AxioCam ICc1 Camera (Zeiss), single and multichannel images were acquired with AxioVision software and analyzed with ImageJ. Dry objectives 10x and 20x were used for SLB adhesion and FRAP studies (Figure 4.7 and 4.8, respectively), and a 100x oil-immersion objective was used for SLB-LUV fusion studies (Figure 4.10).

**4.3. Results and Discussion**

**4.3.1. Design and Synthesis**

This part of the synthesis was carried out by Mingming Ma. Briefly, Tris-(hydroxymethyl)amino-methane) was readily functionalized via a series of known reactions to symmetrically install three terminal chlorides and one terminal carboxylate. Amide coupling of the carboxylate to phosphatidylethanolamine lipid (POPE) yielded a trichloride lipid that was easily transformed to trivalent cyanurate (TCA-PE) or melamine (TM-PE) lipids via thioether formation, with three hydrogen bonding heterocycles per lipid headgroup (Figure 4.1). These lipids were incorporated into synthetic lipid membranes and their ability to guide surface recognition and fusion was evaluated. In addition, the trichloride headgroup was coupled to the N-terminus of the membrane-active peptide magainin; thioether formation with melamine-thiol yielded a peptide conjugate (TMM) that combines both molecular recognition (TM) and membrane activation, in analogy to previously reported systems. We investigated inter-membrane lipid recognition in three different contexts: 1) lipid-lipid binding (TCA-
Fig. 4.1 (A) Schematic illustration of how membrane anchored lipids tri-functionalized with CA or M (symbolized as red and grey wedges) may direct lipid membrane apposition and fusion. (B) Possible modes of inter-membrane hydrogen bonding, based on known CA/M assembly topologies: tape (left) and rosette (right). (C) Structures of compounds used in this study (CA-PE and M-PE have been previously reported). For TMM, the sequence of magainin is shown in bold and K* is lysine (acetamidobenzamide).
PE/TM-PE), lipid-lipid binding with membrane activation by magainin (TCA-PE/TM-PE + mag), and lipid-peptide binding (TCA-PE / TMM). These systems were designed to explore the nature of TCA/TM headgroup binding and the extent of membrane fusion with and without a known disruptive element such as an antimicrobial peptide.

4.3.2. Vesicle-Vesicle Binding

Lipid films were prepared that contained TCA-PE at 0.1 to 5 mole percent in egg phosphatidylcholine (ePC) and TM-PE at the same concentration in 20% phosphatidylglycerol lipid (POPG) and ePC. TM-PE liposomes have a propensity for self-aggregation that is completely suppressed by the inclusion of negatively charged POPG in the preparation. These lipid films were hydrated in phosphate buffer saline (PBS) at pH 7.4 and extruded through 100 nm pore polycarbonate membranes to produce large unilamellar vesicles (LUVs) that appeared to be monodisperse and non-aggregated as judged by dynamic light scattering (DLS). While electrostatically driven binding between oppositely charged membranes and macromolecules in water is well-established, we observe a phenomenon upon mixing of TCA-PE and TM-PE membranes consistent with molecular recognition between neutral components. Notably, despite repulsive potentials of -10 mV and -23 mV for the TCA-PE and TM-PE LUVs (Table 4.1), respectively, mixing the vesicle populations in a 1:1 ratio resulted in rapid doubling of size as judged by DLS (Figure. 4.2). At pH ~7 the cyanuric acid and melamine groups should be neutral, thus the charge on TCA-PE and TM-PE should be -1 due to the phosphate. Replacing TCA-PE and TM-PE with the singly charged POPG yields similar LUV ζ potentials (-12 mV and -23 mV, respectively) (Table 4.1) suggesting that the charge on each lipid is approximately -1, as expected. Additionally,
increasing salt concentration slightly enhances vesicle-vesicle aggregation, consistent with a neutral rather than electrostatic interaction.

**Fig. 4.2.** Size change of vesicles as a function of vesicle aggregation or fusion, measured by DLS. (Top) TCA-PE LUVs reacted with TM-PE LUVs; (Middle) Same as top, in presence of magainin; (Bottom) TCA-PE LUVs reacted with TMM bound to POPG LUVs. Traces represent: TCA-PE (--); TM-PE and TMM/POPG (—); mixed LUVs after 30 minutes equilibration (—).

<table>
<thead>
<tr>
<th>Liposome</th>
<th>TCA</th>
<th>TCA control</th>
<th>TM</th>
<th>TM control</th>
<th>Fused</th>
<th>Fused control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid composition</td>
<td>TCA 5%, ePC</td>
<td>POPG 5%, ePC</td>
<td>TM 5%, POPG</td>
<td>POPG</td>
<td>TCA 2.5%, TM</td>
<td>POPG 15%, ePC</td>
</tr>
<tr>
<td></td>
<td>95%</td>
<td>95%</td>
<td>20%, ePC 75%</td>
<td>25%, ePC 75%</td>
<td>2.5%, POPG</td>
<td>10%, ePC 85%</td>
</tr>
<tr>
<td>ζ Potential (mV)</td>
<td>-12.9</td>
<td>-10.6</td>
<td>-23.4</td>
<td>-23.5</td>
<td>-18.1</td>
<td>-22.4</td>
</tr>
</tbody>
</table>

**Table 4.1.** ζ potentials of TCA, TM modified liposomes, fused liposomes, and controls with competitive surface charge.
4.3.3. Vesicle-Vesicle Fusion

Synthetic vesicle fusion systems driven by molecular recognition between coiled-coil peptides\textsuperscript{33, 34} and proteins derived from the SNARE synaptic vesicle fusion machinery,\textsuperscript{35-38} nucleic acid\textsuperscript{39-41} and small molecules\textsuperscript{9, 24, 30} have been previously reported, as well as systems driven by metal complexation\textsuperscript{42-44} and electrostatics.\textsuperscript{31, 45} We have found that the designed aqueous-phase interaction between TCA/TM-PE lipids can also drive selective membrane merger, though this occurs with contents leakage.\textsuperscript{46} Lipid mixing FRET based assays were used to follow membrane fusion.\textsuperscript{47} We examined three distinct topologies for membrane reactions mediated by TCA and TM: 1) intermembrane lipid-lipid binding, 2) intermembrane lipid-lipid binding with membrane-binding peptide added as a third component, and 3) intermembrane lipid-peptide binding. Though mixing the TCA-PE and TM-PE LUVs resulted in aggregation (Figure 4.2), no lipid mixing was observed, even when a large excess of the non-labeled vesicle was used (Figure 4.3), suggesting that membrane fusion is arrested in the docking stage (Figure 4.4A). Addition of magainin peptide to the docked system triggered rapid lipid mixing, indicating that disruptive peptide-membrane binding may facilitate membrane fusion. The identical experiment was carried out with TMM (Figure 4.1) replacing TM-PE and magainin; reaction of these vesicles with 5% TCA-PE LUVs also resulted in efficient lipid mixing.

Fusion with TMM was concentration dependent, with a minimum surface concentration of 2% required when the reacting membrane contained TCA-PE at 2% (Figure 4.4B). Introduction of the TCA or TM headgroup alone (without membrane anchor) to the TMM fusion system resulted in significant inhibition of lipid mixing,
suggesting that the soluble trivalent headgroups were capable of blocking surface binding sites and suppressing membrane apposition (Figure 4.4C). Interestingly, inhibition with TM was more effective than with TCA, though both elicited a decrease in lipid mixing.

![Graph showing relative NBD fluorescence over time](image)

**Fig 4.3.** Lipid mixing assay on membrane fusion with excess non-labeled TM-PE/POPG/ePC to labeled TCA-PE/ ePC liposomes. (▾) 1:1; (▴) 5:1; (●) 10:1; (■) 20:1.

While the origin of this difference is unclear, the possibility of selective molecular recognition between designed small molecules in aqueous milieu is intriguing; we are investigating these interactions further. We previously reported that LUVs of 100% M-PE and CA-PE fused efficiently, so we compared the efficiency of fusion when the 100% M-PE LUVs were replaced with 5% TM-PE in a POPG membrane. Surface dehydration by hydrogen bonding has not yet been identified as a major mechanism for native mem-
Fig. 4.4. Lipid mixing assay for membrane fusion. All dyes are in CA containing LUV, TM and TCA represent soluble headgroup only, without phospholipid. (A) 3 systems: 5% TMM/POPG/ePC LUVs reacted with 5% TCA-PE/ePC LUVs (●), 5% TM-PE/POPG/ePC LUVs reacted with 5% TCA-PE/ePC and 5% magainin (▲), 5% TM-PE/POPG/ePC reacted with 5% TCA-PE/ePC (◆)); (B) concentration dependence: POPG/ePC reacted with 2% TCA-PE/ePC LUVs at 1% (◆), 2% (▼), 3% (▲), 4% (■) and 5% (●) TMM; (C) inhibition: 3% TMM/POPG/ePC LUVs reacted with 3% TCA-PE/ePC LUVs (●), inhibited with 5 equiv. TCA (---), inhibited with 5 equivalents TM (---), POPG/ePC LUVs reacted with 3% TCA-PE/ePC LUVs with 3% magainin (◆); (D) trivalent/monovalent comparison: 100% M-PE LUVs and 97% CA-PE LUVs with 5% magainin (Δ) and same, without magainin (-Δ-), 5% TM-PE in POPG/ePC LUVs reacted with 97% CA-PE LUVs with 5% magainin (●) and same, without magainin (-●-).

brane fusion, but appears to be an effective strategy that is enhanced when combined with membrane disruption by a peptide anchor. Fusion was observed when 5% TM-PE
LUVs replaced 100% M-PE LUVs, but both reactions were markedly accelerated by addition of magainin peptide as catalyst, yielding similar lipid mixing rates (Figure 4.4D). These findings are consistent with the notion that extensive dehydration is necessary for fusogenic activation via surface H-bonding; TM-PE/CA-PE binding likely results in a smaller contact area and thus requires peptide catalyst for productive docking. With regard to recognition, the similar fusion rates produced by 5% TM-PE and 100% TM-PE in the presence of magainin indicate that the covalent cluster of three melamine rings in TM-PE can produce similar docking effects as an entire surface of M-PE.

4.3.3. Cryo-Electron Microscopy and Freeze-thaw of Docked Vesicles

Hydrogen bonding lipids are known to induce lamellar to hexagonal phase transitions,\(^{48,49}\) as we found with CA/M-PE LUVs.\(^9\) Given the low mole percentage of trivalent lipids in the vesicles studied, we anticipated that the behavior of the membrane should more closely approximate a lamellar phase membrane. Cryo-TEM was used to examine 5% TM-PE and 5% TCA-PE LUVs for signs of hexagonal phase formation and vesicular adhesion before and after mixing.\(^50\) The reactant LUVs alone appeared to be less aggregating populations of LUVs while the images of the LUV mixture consistently indicated lamellar phase LUVs. Vesicular aggregates in the mixture were observed as expected, as well as many structures that appeared to be captured immediately post-fusion, with elongated form approximately twice the diameter of a single LUV and a constriction around the center, possibly from the expansion of a fusion stalk. This observation runs counter to the lack of vesicular fusion detected by fluorescence (Figure 4.4) and suggested that the aggregated vesicles may be meta-stable with regard to fusion.
Fig. 4.5. Lipid mixing assay following freeze-thaw of docked vesicles. (■) TCA-PE/ePC/Rh/NBD LUVs and TM-PE/POPG-ePC LUVs, and controls (♦) TCA-PE/ePC/Rh/NBD LUVs and POPG/ePC LUVs; (▲) POPG/ePC/Rh/NBD LUVs and TM-PE-POPG/ePC LUVs; (▼) POPG/ePC/Rh/NBD LUVs and POPG/ePC LUVs.

We postulated that the sharp and extreme temperature decrease inflicted on the sample by plunging into liquid ethane slush during freezing for cryo-TEM analysis triggered rapid fusion of the vesicles docked by TCA/TM-PE recognition, resulting in the post-fusion vesicle structures. To address this possibility, we formed docked vesicles as previously, with fluorescent lipids to follow lipid mixing, and immersed this sample in liquid nitrogen until frozen. Upon thawing, we found the fluorescence signature of fusion, which was absent in the reactant and unfunctionalized vesicles (Figure. 4.5); multiple freeze-thaw cycles induced fusion in all LUVs, as expected given the cell-membrane rupturing effect of freeze-thaw procedures. Though this experiment does not
duplicate the conditions of cryo sample preparation, the partial fusion resulting from one cycle of freeze-thaw suggests heightened membrane instability in the docked state and supports the notion that cryo-TEM imaging has captured some assemblies shortly after fusion.\textsuperscript{50}

4.3.4. Vesicle Adhesion with Supported Lipid Bilayers

\textbf{Fig.4.6.} Fluorescence microscopy of a supported lipid bilayer (SLB), formed on a fibronectin grid (scale bar= 50 μm) with sharp gradients of Texas Red lipid from left to right and TCA-PE from right to left. (Top) Both sides of the SLB were treated with POPG LUVs containing Oregon Green lipid (OG-PE); (Middle) both sides were treated with TM-PE/POPG LUVs containing OG-PE; (Bottom) OG fluorescence across the SLB for top image (black) and middle image (green).
Supported lipid bilayers (SLBs) are a powerful tool for studying lipid membrane interactions.\textsuperscript{51} We constructed SLBs with a 0-5% concentration gradient of TCA-PE across a surface that was patterned with 50 μm\textsuperscript{2} fibronectin grids that act as diffusion corrals.\textsuperscript{52} These SLBs were used to examine the ability of TCA/TM-PE recognition to direct spatially selective LUV-SLB deposition on the micron scale. Fluorescence microscopy imaging revealed that TM-PE LUV deposition reproduced the surface pattern of TCA-PE with high fidelity, to the extent that adhesion to the unfunctionalized SLB was undetectable (Figure. 4.6). We also monitored deposition of TM-PE LUVs with TCA-PE absent from the bilayer, as well as deposition of simple POPG LUVs onto the TCA-PE SLB; in both cases, no vesicle adhesion was detectable. In fact, adhesion of TM-PE LUVs on the TCA-PE SLB following treatment with POPG LUVs produced an identical result to TM-PE LUV deposition without prior POPG deposition. This data strongly supports the notion of spatially selective membrane apposition driven by binding of TCA-PE and TM-PE.

The adhered LUVs (labeled with TR-DHPE) exhibited markedly less lipid mobility than the SLB. While the SLB fluorophore exhibited significant fluorescence recovery after photobleaching (FRAP), the adsorbed layer did not recover after extended monitoring (Figure. 4.7). This differential mobility suggests the absence of SLB-LUV fusion, as expected from vesicle-vesicle fusion experiments (Figure. 4.6) though it is possible that the FRAP experiment is less sensitive to this process. Vesicle fusion with the SLB should lead to similar FRAP in both channels; as it is not observed with vesicle-bound TR-DHPE, then it is likely that TR-DHPE is part of an unfused, multivalently tethered vesicle. The numerous multivalent TCA/TM-PE interactions clustered at the
SLB/LUV interface present a significant diffusion barrier, unlike DNA-tethered vesicle systems, possibly due to higher surface concentration. The requirement for FRAP in the adhered LUV layer (assuming no membrane fusion) is essentially desorption/resorption, and this process is extremely slow. Thus, it is likely that the hydrogen bonding lipids involved in the surface contact on both the LUV and SLB are immobile while the fluorescently labeled lipids remain mobile in the continuous SLB.

**Fig. 4.7.** Fluorescence microscopy of a vesicle-bound SLB on a 50 μm feature fibronectin grid. The SLB was labeled with OG-PE and TCA-PE; vesicles were labeled with TR-PE and TM-PE. Surface was photobleached and FRAP observed in both the SLB (green) and vesicle (red) layers. (Left) OG-PE fluorescence channel immediately after photobleaching; (Left, Center) 4 minutes after; (Right, Center) TR-PE channel immediately after photobleaching; (Right) 4 minutes after.

**4.3.5. Vesicle Fusion with Supported Lipid Bilayers**

LUV-SLB fusion was studied using the same lipid mixing FRET assay as in suspension. Treatment with TM-PE or TMM functionalized LUVs should result in vesicle docking and fusion, depending on conditions (Figure 4.4). Lipid mixing dilutes the surface bound NBD-PE/Rh-DHPE FRET pair and dequenches the FRET donor (NBD); we monitored both NBD and Rh fluorescence channels and evaluated fusogenic conditions using ten experiments (A-J, Table 4.2). SLB imaging generally tracked with
LUV-LUV suspension results. Both TCA/TM recognition groups had to be present to produce a lipid mixing signal while control experiments (A, C, E, G-J, Table 4.2) yielded minimal signal changes (Figure 4.8). Replacement of recognition groups with simple charges did not result in detectable reaction, again ruling out electrostatic interactions as the main driver of binding. One notable deviation from LUV-LUV and LUV-SLB experiments (Figure 4.4 and 4.6) was the positive fusion signal observed with TM-PE and TCA-PE interactions. Our previous experiments indicated that this system exhibited surface adhesion without fusion and vesicle fusion experiments did not even suggest partial lipid mixing that could be attributed to hemifusion. Under LUV-SLB fusion conditions (Figure 4.8 and 4.9F), strong lipid mixing was observed. This again points towards the meta-stability of the docked system with regard to fusion or hemifusion (these experiments do not distinguish the two), as suggested by cryo-TEM data. Variation in stoichiometry, lipid composition and fluorophore system between LUV-SLB fusion, LUV-LUV fusion and LUV-SLB adhesion (Figure 4.4 and 4.6) experiments could cause a range of membrane reactivity and detectable response. Overall, these data indicate a lipid recognition system in which there is only a slender mechanistic separation of surface recognition and membrane activation and merger, thus minor perturbations in the docked system may spontaneously trigger disruption and lipid mixing.
Table 4.2. Conditions for LUV-SLB reaction

<table>
<thead>
<tr>
<th>SLB (in ePC)</th>
<th>LUV (in ePC)</th>
<th>Lipid mixing</th>
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<tbody>
<tr>
<td>A</td>
<td>5% POPG 25% POPG, 1% TMM</td>
<td>(-)</td>
</tr>
<tr>
<td>B</td>
<td>5% TCA-PE 25% POPG, 1% TMM</td>
<td>(+)</td>
</tr>
<tr>
<td>C</td>
<td>5% TCA-PE 25% POPG, 1% Mag</td>
<td>(-)</td>
</tr>
<tr>
<td>D</td>
<td>5% TCA-PE 20% POPG, 1% Mag, 5% TM-PE</td>
<td>(+)</td>
</tr>
<tr>
<td>E</td>
<td>5% POPG 25% POPG</td>
<td>(-)</td>
</tr>
<tr>
<td>F</td>
<td>5% TCA-PE 20% POPG, 5% TM-PE</td>
<td>(+)</td>
</tr>
<tr>
<td>G</td>
<td>5% TCA-PE 1% TMM alone</td>
<td>(-)</td>
</tr>
<tr>
<td>H</td>
<td>5% TCA-PE 1% Mag alone</td>
<td>(-)</td>
</tr>
<tr>
<td>I</td>
<td>5% POPG 20% POPG, 1% Mag, 5% TM-PE</td>
<td>(-)</td>
</tr>
<tr>
<td>J</td>
<td>5% POPG 25% POPG, 1% Mag</td>
<td>(-)</td>
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</tbody>
</table>

Fig. 4.8. Total integrated NBD fluorescence change on SLB surface after 20 minutes of reaction and surface washing. Experimental conditions are shown in Table 4.2. All conditions have 1.5% each NBD-PE and Rh-PE in the SLB. Reactions and measurements were performed in triplicate.
Fig. 4.9. (Top) Schematic illustration of vesicle-SLB fusion, followed by dilution of NBD/Rh FRET in the SLB. (Below) NBD fluorescence microscopy of SLBs containing 2% NBD-C6 HPC, 2%Rh-DHPE in ePC. Images A-F correspond to conditions described in Table 4.2.
4.4. Conclusions

Our examination of designed lipid recognition at the bilayer-water interface has yielded findings along two fundamental lines: molecular recognition and membrane merger. While selective membrane fusion requires both recognition and disruption, these functions need not be performed by the same molecule. Trivalent lipid-lipid binding can induce membrane apposition, but in the vesicle-vesicle context, no fusion or lipid mixing. Addition of a membrane-disrupting peptide as a third component (TCA-PE + TM-PE + Mag) results in lipid mixing with high efficiency (Figure. 4.4 and 4.9). Similarly, fusion was observed when one of the components was membrane anchored with the same peptide (TCA-PE + TMM). Given the fusogenicity of the 3-component system, it appears that membrane activation need not be precisely at the site of molecular recognition. Though the surface concentration of H-bonding trivalent lipids is relatively low, the docked vesicular aggregate is unstable with regard to fusion, possibly due to headgroup H-bonding which facilitates the formation of non-bilayer fusion intermediates.48, 49 Indeed, the meta-stability of TCA/TM lipid docked membranes may explain why fusion not observed in LUVs, but observed in LUV-SLB binding and possibly during cryo-TEM sample preparation. Though mechanistic questions remain, experiments further illustrate the general principles of membrane merger and demonstrate a new designed functional recognition motif. These studies have revealed that low membrane concentrations of the trivalent CA/M lipids or peptides retain the robust molecular recognition properties found with surfaces composed entirely of monovalent CA/M recognition lipids, though membrane activation upon binding is diminished. The minimal nature of headgroup design and synthetic simplicity stands in contrast to the
effectiveness of recognition function. Recognition appears to occur between neutral components: though it is conceivable that a pKa shift induced at the lipid-water interface could generate a negatively ionized cyanuric acid and a positively charged melamine, we find that the contribution of electrostatic interactions is minor to non-existent in our membrane-anchored TCA/TM system. Thus we conclude that the main driving force for recognition is not charge complementation, but rather, more subtle hydrogen-bond donor-acceptor pattern recognition between largely neutral species in competing aqueous solvent. Moreover, this interaction is sufficiently strong to overcome hydration repulsion forces\textsuperscript{55} between membranes to induce both spatially selective surface adhesion and membrane merger. The biophysics of this binding interaction in water, as well as the range of aqueous-phase assembly chemistry that may be mediated by cyanurate and melamine groups, remains an intriguing topic with potential relevance to the development of new functional materials.

4.5. References for Chapter 4
CHAPTER 5

LIPID PROBES FOR IDENTIFICATION OF PHOSPHATIDYL SERINE RECEPTORS AND CELL UPTAKE OF PHOSPHOSERINE POLYMERS

5.1. Introduction

The clearance of apoptotic cells by phagocytes is a prerequisite to restore normal tissue function and plays a critical role in the resolution of inflammation\(^1\)-\(^3\). It ensures routine turnover rate of cells and protects tissues from exposure to the inflammatory or immunogenic contents released from dying cells during their lysis. As cells undergo apoptosis, they are rapidly phagocytosed by professional phagocytes such as macrophages and dendritic cells through a multi-step process which includes phagocyte attraction, recognition, engulfment and digestion (Figure 5.1). In this neatly regulated, yet not fully understood clearance process, specific recognition of apoptotic cells by phagocytes is an essential step. This recognition is generally thought to be facilitated by apoptosis-associated cell membrane alteration. While many other changes such as altered carbohydrates\(^5\), oxidized LDL-like moieties\(^6,7\), or serum derived proteins\(^8\) exist, a crucial alteration on the membrane is the exposure of phosphatidylserine (PS)\(^9\). In the membrane of healthy cells, PS is usually restricted to the inner leaflet. One of the best studied mechanisms indicates that phospholipid asymmetry is achieved by aminophospholipid
translocase, which flips PS back to the inner leaflet once it appears outside\textsuperscript{10}. Translocase activity is disrupted during the process of apoptosis, resulting in exposure of PS on the outer cell surface. The PS “eat-me signals” are then recognized by PS receptors on phagocytes, which initiates the process of clearance of apoptotic cells.

![Diagram of apoptosis and phagocytosis](attachment:image.png)

**Fig. 5.1.** Elimination of unwanted cells in vivo. Unnecessary or harmful cells, such as cancer cells or virus-infected cells, are eliminated by apoptosis. Apoptotic cell corpses express "eat-me signals" on their surface, and are rapidly recognized and phagocytosed by professional phagocytes, including macrophages and dendritic cells. Figure adapted from reference 11.

While the engulfment of pathogens results in stimulation of immune response and accompanying inflammation, apoptotic cell clearance by phagocytes does not induce pro-inflammatory responses; rather, it produces of anti-inflammatory cytokines such as TGF-β1 and PGE2 and the suppresses the release of pro-inflammatory mediators, including IL-8, TNF-a and TXA2, from activated\textsuperscript{12, 13}. Consequently, clearance of apoptotic leukocytes is involved in the resolution of inflammation and accumulating discoveries indicate that defective clearance of apoptotic cells contributes to inflammatory and autoimmune diseases. Finding the corresponding receptors on phagocytes, with which
they involve in PS recognition, not only helps understand this critical process but also is to benefit the development of novel anti-inflammatory drugs.

Our first objective is to develop lipid probes for efficient identification of new PS receptors. In this project, synthetic PS-lipid probes will be used to screen multiple cell types for novel PS receptors in an unbiased fashion. Affinity probes used in activity based protein profiling (ABPP) have had great success and has emerged as a powerful chemical proteomic strategy in studying numerous native biological systems,¹⁴ there have not been any previously reports of PS-affinity probes.

![Fig. 5.2. Representative structure of an affinity probe, which contains a reactive group (green), a binding group, or spacer (black), and a reporter tag (purple). Modified from reference 14.](image)

The basic building blocks of affinity probes used in literature typically contains: a binding motif that recognizes its target; a reactive group that covalently attaches the probe to what it binds to; and a reporter tag to facilitate target characterization; in some cases, there are also spacer groups that optimize the selectivity. There are generally two types of reactive groups: photoreactive groups that label proximal residues in a protein following UV irradiation and electrophilic groups that covalently modify conserved binding-site nucleophiles. A reporter tag can be biotin, fluorophores, or azides which can...
be modified later by “click” chemistry for enrichment, visualization, or characterization purposes.

One example of ABPP by the Cravatt group is described here. In that work, they were able to characterize a large, diverse class of enzymes called metalloproteases involved in many physiological and disease processes, which was normally ineffectively characterized by conventional genomic and proteomic methods. The metalloprotease-directed probe was designed based on its Hx inhibitors; the core structure of these reagents was derivatized with a benzophenone (BP) photo-cross-linker and an alkyne group (Figure 5.3). In gel-based ABPP, individual HxBPyne-treated proteomes are reacted with an azide-rhodamine (Rh) fluorescent reporter tag under click chemistry conditions and separated by SDS-PAGE. Labeled metalloproteases are visualized by in-gel fluorescence scanning. In ABPP-MudPIT, proteomes are treated with a cocktail of HxBPyne probes and reacted with an azide-biotin (B) reporter tag under click chemistry conditions. Probe-labeled metalloproteases are then captured on avidin beads, digested with trypsin and analyzed by multidimensional LC-MS.

Our overall strategy for labeling new PS receptors in multiple cell lines is described below (Figure 5.4). For initial testing of the probe, fluorescence reporting tags such as Rh can be used to compare selectivity between designed probe and control probes using SDS-PAGE separation and in-gel fluorescence scanning. Once the selectivity is verified, the reporter tag can be replaced by biotin, thus making enrichment of labeled proteins on avidin beads possible. This enrichment strategy can provides the opportunity of protein identification via LC-MS. PS binding sites within the receptor may be further indentified by similar digestion strategies as demonstrated in Figure 5.3.
Our second objective is to develop a PS modified polymer as an anti-inflammatory drug candidate. Macrophage engulfment of apoptotic cells induces TGF-β1 secretion, leading to an anti-inflammatory effect and suppression of pro-inflammatory mediators. Researchers\textsuperscript{17} have shown that direct instillation of PS expressed apoptotic cells or PS liposomes that induce TGF-β1 locally, enhanced the resolution of acute inflammation. Cells that do not express PS on their surface, such as viable or opsonized apoptotic Jurkat cells, failed to induce TGF-β1. As PS liposomes face the problem of stability when used as drugs, polymers that are modified by PS headgroups may function the same while being stable in circulation, thus providing a novel approach to treatment of inflammation.
Fig. 5.4. Schematic illustration of the PS-labeling strategy. Multiple cell lines or lysates will be incubated with tag-free, benzophenone (BP) and alkyne conjugated PS probes. Fluorescent reporter tags will be coupled selectively to these labeled proteins by click chemistry, thus the resulting conjugate can be separated by SDS-PAGE and analyzed by in-gel fluorescence scanning. Illustration modified from reference 16.

Our initial design of the PS polymer contains a negative polymeric back chain, partially modified by PS (Figure 5.5), and fluorescent tag such as FITC, which can be conjugated to the polymer by the primary amine groups. The uptake of these PS polymers will be compared between cell lines bearing different amount of PS receptors on their surface, as followed by FACS. Secretion of TGF-β1 as a result of uptake will be verified by Western blot, ELISA, or other cell assays.
Fig. 5.5. Structure of PS-Polymer. FITC can be attached to the polymer at * marked amine at a certain percentage, resulting in the polymer FITC-PS-Polymer.

5.2. Materials and Methods

Chemicals and reagents. All chemicals, proteins, antibodies and other reagents were used as provided by the manufacturer unless otherwise indicated.

Synthesis. All synthetic work in this chapter was done by Dr. Saibal Bandyopadhyay in our group.

Cell culture. Detailed culture conditions for each cell line involved in this chapter are described in Chapter 6.

PS-FITC binding experiments as in Figure 5.7. Four cell lines: Raw264.7, THP-1, Jurkat, M210B4 were used. 1 million cells of each cell line was incubated with buffer, FITC-PS (5 μM), or FITC-PS (5 μM) and competitor PS (50 μM) in 200 μl HBSS buffer with calcium for 30 minutes at 37°C. Cells were washed twice and fixed in 2% paraformaldehyde for FACS.

Labeling experiments in Figure 5.9. Jurkat cells were freeze/thawed in HBSS with 0 μM or 50 μM calcium and total protein concentration was adjusted to 2.6 mg/ml. To 38 ul of this lysates was added BPPS (2.1 μM or 21 μM, 2 μl), PS (200 μM, 2 μl) or
HBSS (2 μl to balance the volume between samples). The solution was mixed and incubated for 15 minutes at RT. They were then transferred on ice and irradiated by UV at 365 nm for 1 hour. Rh azide was added (420 μM, 3 μl), followed by the addition of click reaction cocktailtails (a) 5 μl solution containing CuSO₄ (1 mM) and sodium ascorbate (10 mM), or (b) TCEP (50 mM, 1 μl), ligand (1.7 mM, 3 μl) and CuSO₄ (50 mM, 1 μl). The mixtures were then incubated at RT for 1 hour. Each sample was centrifuged and re-dissolved in 40 μl HBSS, followed by precipitation using 150 μl methanol, then re-dissolved in HBSS for SDS-PAGE.

**Labeling experiments in Figure 5.10.** THP-1 monocytes in HBSS were fractionated as described in chapter 6 and protein concentration adjusted to 4 mg/ml by Bradford assay. Labeling reactions and methanol precipitation were carried out in the same way as described above.

**Labeling experiments in Figure 5.11.** 15 million/ml THP-1 monocytes were used instead of lysates and all reactions were scaled up in order to provide enough volume for sonication. Reaction mixtures were not precipitated by methanol but instead the SDS gel was ran for a prolonged-10 mins to avoid contamination by Rh azide successfully.

**Labeling experiments in Figure 5.12.** THP-1 monocytes were differentiated to macrophages as described in chapter 6. Whole cell lysate was used and labeling reactions were carried out as in Figure 5.9. No methanol workup.

**Cellular uptake of PS-polymer in Figure 5.13.** THP-1 monocytes were differentiated to macrophages as described in chapter 6. They were serum starved for 1 hr at 0.5 million cells per ml in DPBS. FITC-PS-Polymer or FITC dextran was added,
followed by 2 hour incubation. Cells were digested by trypsin, washed with DPBS and fixed by 2% paraformaldehyde for FACS.

5.3. Results, Discussion and Outlook

5.3.1. Labeling Experiments

5.3.1.1. Use FITC-PS to Probe Membrane PS Receptors

![A](image)

![B](image)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Morphology</th>
<th>Expected PS Receptor density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw 264.7</td>
<td>macrophage</td>
<td>high</td>
</tr>
<tr>
<td>THP-1</td>
<td>monocyte</td>
<td>high</td>
</tr>
<tr>
<td>Jurkat</td>
<td>lymphoblast</td>
<td>low</td>
</tr>
<tr>
<td>M2-10B4</td>
<td>fibroblast</td>
<td>low</td>
</tr>
</tbody>
</table>

Fig. 5.6. A. structure of FITC-PS-C4; B. structure of PS-C4; C. information of the four cell lines used in the experiment.
As a preliminary test, FITC-PS-C4 (Figure 5.6A) conjugate was synthesized and used to test its ability binding to PS receptors on the cell membrane. PS-C4 (Figure 5.6B) was used as a competitor molecule to examine if the interaction is PS-receptor guided or non-specific. Four cell lines (Figure 5.6C): Raw264.7, THP-1, Jurkat, M210B4 were used. However, our result (Figure 5.7) shows that binding of FITC-PS-C4 is not inhibited by the presence of PS-C4, suggesting that the binding is non-specific.

We used fluorescence anisotropy to further probe the binding activity of FITC-PS-C4, annexinV, a known PS receptor protein was titrated to FITC-PS-C4, however, no significant change (Table 5.1) in anisotropy was observed under our experiment conditions while annexin V is known to bind FITC-PS-C4 in nM range. It is unclear why this experiment failed.

**Fig. 5.7.** FACS result of FITC-PS binding experiments. Y-axis displayed medium FITC fluorescence intensity of treated cells as determined by FACS.
Table 5.1. Fluorescence anisotropy results on binding between FITC-PS and annexin V.

<table>
<thead>
<tr>
<th>FITC-PS</th>
<th>Annexin V</th>
<th>Anisotropy</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 nM</td>
<td>0</td>
<td>0.020</td>
</tr>
<tr>
<td>100 nM</td>
<td>270 nM</td>
<td>0.019</td>
</tr>
<tr>
<td>500 nM</td>
<td>0</td>
<td>0.020</td>
</tr>
<tr>
<td>500 nM</td>
<td>540 nM</td>
<td>0.016</td>
</tr>
<tr>
<td>500 nM</td>
<td>1.08 uM</td>
<td>0.017</td>
</tr>
<tr>
<td>1 uM</td>
<td>0</td>
<td>0.017</td>
</tr>
<tr>
<td>1 uM</td>
<td>1.08 uM</td>
<td>0.017</td>
</tr>
</tbody>
</table>

5.3.1.2. Labeling of PS receptors in cell lysates or live cells using photo reactive PS probes

In the first experiment, probe BPPS (Figure 5.8A) was tested with or without the competitor molecule PS (Figure 5.6B). These reactions with Jurkat cell lysate was also carried out under different conditions: with or without the presence of calcium in HBSS buffer; BPPS concentrations; click reaction conditions. The results from these experiments (Figure 5.9) show that: (a) PS does not inhibit binding of BPPS, (b) new bands are labeled in the presence of calcium, (c) labeling efficiency is BPPS concentration dependent, (d) the TCEP/Ligand/Cu condition works better in these experiments.

We introduced a control molecule for BPPS, BPCOOH (Figure 5.8B) for the following labeling experiment, which is done to fractionated THP-1 cell lysates in vitro. Labeling results with BPPS or BPCOOH were compared on the whole lysate, soluble
fraction and membrane fraction. Another set of experiments was done by denaturing the reaction mixtures after UV irradiation (Fig. 5.10). While denatured samples gave similar results, some proteins were labeled by BPPS but not by the control BPCOOH,
these proteins may potentially be PS receptors. We can also see that these different protein exits mainly in the membrane fraction, consistent with the notion that PS receptors are displayed on cell surfaces.

![Image](image.png)

**Fig. 5.10.** Rh fluorescence image of SDS gel of the *in vitro* labeling on fractionated THP-1 cell lysate.

In this next experiment, we ran the labeling reactions with live cells which were sonicated at different steps: before UV irradiation (same as the *in vitro* experiment discussed earlier), before click reaction, or after the click reaction (Figure 5.11). Labeling experiments with these intact cells resulted in a different profile compared to *in vitro* experiments with less noticeable selective labeling of BPPS compared to BPCOOH. Incubation *in vivo* had the problems in keeping cells well suspended during incubation.
and a lowered labeling efficiency due to accessibility of the proteins while the cells are intact. These problems need to be addressed in future experiments.

![Image of SDS gel](image)

**Fig. 5.11.** Rh fluorescence image of SDS gel of the *in vivo* labeling on THP-1 whole cell lysate.

When THP-1 monocytes are treated with phorbol myristate acetate (PMA) which differentiates them into macrophage cells, there should be an increased amount of PS receptors on their membrane compared to the untreated monocytes. Thus we compared the labeling of these two cells (Figure 5.12). However, no change was observed while macrophage cell lysates were labeled, while the difference between labeling with BPPS and BPCOOH still exits. It indicates that either the differentiation was not successful, which is unlikely due to the observed morphology change of the cells; the amount of PS-receptors do not differ significantly between the two cells; or, the affinity probes were not
labeling PS-receptors selectively. Again, all these possibilities need to be researched in the future.

Fig. 5.12. Rh fluorescence image of SDS gel of the labeling on THP-1 monocytes (marked as T) or THP-1 macrophages (marked as mT) cell lysates.

Proteins we discovered that are labeled by BPPS but not BPCOOH need to be conjugated by biotin azide, followed by enrichment using avidin beads. Their identity may then be revealed by LS/MS and further confirmed if they are PS-receptor proteins, thus validating our mythology of labeling.

5.3.2. Cellular Uptake of Novel PS Polymers

We observed cellular uptake of FITC-labeled PS-polymer (FITC-PS-Polymer, Figure 5.5) by macrophage THP-1 cells under fluorescence microscopy (Figure 5.13) and by FACS (data not shown). FITC-PS-Polymer was incubated with monocytes THP-1 or macrophage THP-1 cells. Control experiments were done by replacing FITC-PS-
polymer by FITC-dextran with a molecular weight of 40kDa. FCAS results (Figure 5.14) showed significantly higher uptake efficiency by macrophage THP-1 cells while the control FITC-dextran did not bind to neither cells selectively. This result is promising even though, better control that bares the same charge as the FITC-PS-Polymer should be made and tested. Also, whether this uptake process evolved cytokine, such as TGF-β1 production needs to be confirmed.

Fig. 5.13. Macrophage THP-1 cells uptake of FITC-PS-Polymer. Left: treated cells; right: control, untreated cells.

Fig. 5.14. FACS results. Cellular uptake of FITC-PS-Poly or FITC-dextran.
5.4. References for Chapter 5

CHAPTER 6

PROTOCOLS

6.1. Fusion Assays

6.1.1. Lipid Mixing Assays

Struck, Hoekstra and Pagano introduced lipid-mixing assays based on NBD–rhodamine energy transfer.\(^1\) In this method (Figure. 6.1), membranes labeled with a combination of fluorescence energy transfer donor and acceptor lipid probes — typically 1.5 mole % NBD-PE (N360, Invitrogen) and 1.5 mole % N-Rh-PE (L1392, Invitrogen), respectively — are mixed with unlabeled membranes. Change in fluorescence resonance energy transfer (FRET), detected as rhodamine emission decrease at ~585 nm (usually 589 nm on our Perkin-Elmer LS50B) or NBD emission increase at ~525 nm (usually 527 nm on our Perkin-Elmer LS50B) resulting from NBD excitation at ~470 nm, can be observed when the average spatial separation of the probes is increased upon fusion of labeled membranes with unlabeled membranes. The reverse detection scheme, in which FRET increases upon fusion of membranes that have been separately labeled with donor and acceptor probes, has also proven to be a useful lipid-mixing assay.\(^2\)
Fig 6.1. Pictorial representation of a lipid-mixing assay based on fluorescence resonance energy transfer (FRET). The average spatial separation of the donor (D) and acceptor (A) lipid probes increases upon fusion of labeled membranes with unlabeled membranes, resulting in decreased efficiency of proximity-dependent FRET (represented by yellow arrows). Decreased FRET efficiency is registered by increased donor fluorescence intensity and decreased acceptor fluorescence intensity. Illustration adapted from Invitrogen catalog.

6.1.2. Content Mixing Assays

6.1.2.1. ANTS/DPX Assay

Originally developed by Smolarsky and co-workers\(^3\) to follow complement-mediated immune lysis, the ANTS/DPX fluorescence quenching assay has since been widely used to detect membrane fusion\(^4\). This assay is based on the collisional quenching of the polyanionic fluorophore ANTS by the cationic quencher DPX (Figure. 6.2). Separate vesicle populations were loaded with a) 25 mM ANTS (Invitrogen), 40 mM NaCl, buffered with 10mM Tris and b) 90 mM DPX (Invitrogen) buffered with 10mM at pH 7.4. Liposomes (5mM, 400ul) after extrusion were passed through a G25( sigma) size exclusion column (20cm column, packed ~90% full) with an elution buffer c) 10mM Tris and 150mM NaCl at pH 7.4, cloudy fractions were collected, resulting in ~1mL of...
diluted liposome solution. Lipid concentrations can be determined by method described in 7.1.4.

![Pictorial representation of the ANTS/DPX vesicle-fusion assay. Illustration adapted from Invitrogen catalog.](image)

**Fig.6.2.** Pictorial representation of the ANTS/DPX vesicle-fusion assay. Illustration adapted from Invitrogen catalog.

### 6.1.2.2. Tb^{3+}/DPA Assay

In the Tb^{3+}/dipicolinic acid (DPA) assay, which was originally described by Wilschut and Papahadjopoulos⁵, separate vesicle populations are loaded with a) 8 mM TbCl₃ (Invitrogen), 60 mM sodium citrate, buffered with 10mM Tris or b) 50 mM DPA, 20 mM NaCl buffered with 10mM Tris at pH 7.4. Liposomes (5mM, 400 μl) after extrusion were passed through a G25 (sigma) size exclusion column (20 cm column, packed ~90% full) with a elution buffer c) 10 mM Tris, 100mM NaCl, 1 mM EDTA at pH 7.4, cloudy fractions were collected, resulting in ~1 mL of diluted liposome solution. Vesicle fusion results in formation of Tb^{3+}/DPA chelates that are ~10,000 times more
fluorescent than free Tb$^{3+}$ (Figure 6.3). Fluorescence of the chelates is detected at 490 nm or 545 nm, with excitation at 276 nm.

![Diagram of Tb$^{3+}$/DPA fluorescence enhancement assay for vesicle fusion. Illustration adapted from Invitrogen catalog.]

**Fig.6.3.** Pictorial representation of the Tb$^{3+}$/DPA fluorescence enhancement assay for vesicle fusion. Illustration adapted from Invitrogen catalog.

### 6.1.3. Leakage Assays

Carboxyfluorescein (Cbf) was encapsulated in liposomes at a 30-50 mM concentration with buffer, and after extrusion they were passed through a gel column as described earlier, equilibrated with another buffer that has the same osmotic pressure compared to the buffered Cbf solution. The orange fraction which comes out the first was collected and this fraction should be well separated from the free dye which comes off afterwards with a clear band in between on the column. Liposomes should be used as soon as they are prepared.
6.1.4. Fluorimeter Usage Instructions

For detailed instrument manual, refer the “LS50B User’s Guide” included in the “Manuals” folder in the CD attached.

General Procedures:

1. Turn on water bath, set temperature, this will be water circulation temperature; turn on pettir, set temperature, this will be corvette temperature; turn on computer; turn on Perkin-Elmer LS50B; open software XWINlab on desktop.
2. Click on “utilities”, change data saving settings to your desired data folder, click OK. You can also change data saving formats in this window.
3. Click the measurement icon; click on “method open” in the measurement window, open your desired method. Always check settings before measurements.
4. Start measurements.
5. You can find readings at a specific wavelength; zoom in/out spectrums; hide spectrums; etc. by using tools on top of the measurement window.
6. You may also view previous data traces by clicking on View-Add Curves in the main window.
7. When you are done, close XWinLab, turn off the fluorimeter, turn off the peltier system, then turn off water bath. DO NOT LEAVE PELTIER ON ALONE, because it continues heating, and without the cooling water bath, the cell holder will melt.

6.2. Supported Lipid Bilayers
6.2.1. SLB Formation

Glass slides or cover slips are boiled in detergent solution (Linbro 7X Lab Glass Cleaner, ICN Pharmaceuticals) for 20 minutes, follow by extensive rinsing overnight, and then bake at 450 °C for 4 hours. Supported lipid bilayers are formed by exposing glass slides or cover slips to a sample of desired liposomes (typical lipid concentration higher than 0.25 mM) for 5 minutes follow by rinsing with excess DPBS buffer, keeping lipid bilayer hydrated at all times.

6.2.2. Microcontact Printing and Y Channel Formation

Poly(dimethylsiloxane) (PDMS) stamps for microcontact printing are formed by curing Sylgard 184 (Dow Corning) on a silicon master (a generous loan from Steven G. Boxer, Stanford University) with patterned photo-resist. Stamps (1x1 cm) are cleaned in a plasma cleaner (Harrick Plasma) for 20 seconds at the highest power setting with a slight leak to room air. The patterned side is soaked immediately with 50-100 μl of 100 μg/ml fibronectin (Sigma) in DPBS buffer for 20 minutes, then dried under a stream of nitrogen. Stamping is done by inverting the stamp and placing it on a cleaned glass slide or cover slip with a 40g weight on top for 20 minutes. After that the glass slide is rinsed with buffer, water, and is then dried under a stream of nitrogen. For FRAP experiments, a chamber gasket (Invitrogen) is adhered on top of the patterned glass surface (Figure 6.4); for experiments involving the converging channels, two layers of PDMS elastomer are cut using a template and layered to produce three walls of the converging channel. Channel is assembled on top of the patterned glass, which comprised the fourth wall of the channel.
6.2.3. Simplified FRAP Experiments

A round spot on a SLB is bleached by a beam of excitation light and pictures are taken after a certain amount of time to observe fluorescence recovery. Typically, the bright 20 x lens is used for a faster bleaching. Bleaching time depends on the dye used and the SLBs, and can be adjusted according to experimental outcomes. For example, it usually takes 30 seconds to bleach a spot well on a NBD SLB. Use highest energy possible to bleach and use lowest energy or exposure time possible to take recovery pictures in order to avoid further bleaching during recovery.

6.3. Cell Culture, Labeling and Uptake Experiments

6.3.1. General Techniques for Cell Culture

6.3.1.1. Aseptic Technique

1. Turn on the laminar flow hood and the UV light. Leave on for 15-30 minutes.

   Turn off UV and open the window. Let it flow for 15 minutes.

2. Start with a completely clear surface. Swab the surface with 70% alcohol.
3. Bring onto the surface only those items you require for a particular procedure. Swab the items carefully before entering the hood.

4. Remove everything that is not required, and swab the surface down between procedures.

5. Arrange your work area so that you have:
   a. Easy access to all items without having to reach over to get at another
   b. A wide, clear space in the center of the bench (not just the front edge!) to work on. If you have too much equipment too close to you, you will inevitably brush the tip of a sterile pipette against a nonsterile surface, and the laminar airflow will fail in a hood that is crowded with equipment

6. Work within your range of vision (e.g. insert a pipette in a pipetting aid with the tip of the pipette pointing away from you so that it is in your line of sight continuously and not hidden by your arm)

7. Mop up any spillage immediately and swab the area with 70% alcohol

8. Remove everything when you are finished, and swab the work surface down again.

9. Shut the window and turn off the hood.

6.3.1.2. Incubators

1. Should be cleaned out at regular intervals (weekly or monthly, depending on access frequency) by removing the contents, including all the shelves with a nontoxic detergent such as Decon or Roccall. Traces of detergent should then be removed with 70% alcohol, which should be allowed to evaporate completely before replacing the shelves and cultures.
2. A fungicide, such as 2% Roccall or 1% copper sulfate, may be placed in the humidifier tray at the bottom of the incubator to retard fungal growth. Refill or replace the water tray so that it is always free of contamination and maintain 1/2 to 2/3 full. Empty water tray will cause the CO₂ sensor fail to work.

3. Check the CO₂ level in the tanks at least once a week.

4. Overlook the incubator daily in minimum.

6.3.1.3. Medium Preparation

1. Avoid repeating freeze/thaw circles on serum and antibiotics. Order small packing or split them into aliquots after first use. Label tubes with product name, catalog number, preparation date and expiration date.

2. Thaw serum and antibiotics in 4degree fridge 2 days before medium preparation. L-Glutamine needs to be incubated in 37°C water bath for 30 minutes immediately before use because of solubility issues. We used heat-inactivated FBS from Sigma.

3. Prepare medium according to ATCC guidelines for each cell line. Label bottle with its components, preparation date, your name and the cell line it is used for. Medium should be prepared freshly every 2-3 weeks.

6.3.1.4. Cryopreservation and Thawing Frozen Cells

Optimal freezing of cells for maximum viable recovery on thawing depends on minimizing intracellular ice crystal formation and reducing cryogenic damage from foci of high-concentration solutes formed when intracellular water freezes. This is achieved by
a. Freezing slowly to allow water to leave the cell but not so slowly that ice crystal growth is encouraged

b. Using a hydrophilic cryoprotectant to sequester water

c. Storing the cells at the lowest possible temperature to minimize the effects of high salt concentration on protein denaturation in the micelles within the ice

d. Thawing rapidly to minimize ice crystal growth and generation of solute gradients formed as the residual intracellular ice melts

**Cryopreservation**

1. Determine cell concentration according to ATCC or based on the rule that the number of cells frozen should be sufficient to allow for 1:10 dilution on thawing but still keep the cell concentration higher than at normal passage.

2. Grow the culture to late log phase before entering plateau and, if using a monolayer, trypsinize and count the cells; if using a suspension, count and centrifuge the cells.

3. Resuspend cells in complete growth media to twice as much as the desired cryopreservation concentration. Dilute the cell suspension 1:1 with a freezing media containing twice of desired DMSO amount according to ATCC.

4. Dispense the cell suspension into pre-labeled ampoules, and cap the ampoules with sufficient torsion to seal the ampoule without distorting the gasket.

5. Place the ampoules on a Nalge Nunc Cooler filled with IPA pre-warmed to room temperature. Place this Cooler in -80 freezer overnight to let the cell to freeze at a rate of 1 degree per minute.
6. Transfer the ampoules to a liquid N\textsubscript{2} freezer quickly. Record on log book the number and location of ampoules.

Thaw

1. Transfer the ampoules from liquid N\textsubscript{2} to a 37 degree water bath as fast as possible and slightly twist the lid without contaminating the cells to let out any liquid N\textsubscript{2}. The whole ampoule should be thawed completely within 2 minutes.

2. Add the 1ml cell solution to 10-14ml warmed media drop wise with caution, and spin it at 150g for 5 minutes. Remove the media and replace it with desired amount of fresh media to a certain cell density, then transfer to culture flasks/plates. When thawing cells you can grow them in a media contains up to 20\% serum if needed, for the first few days.

3. Keep tracking the cells at least daily until they grow normally. It usually takes up to a week for this depending on the cell line.

6.3.1.5. Ordering Information for Cell Culture

Refer the “Ordering Information for Cell Culture” included in the attached CD labeled “Ordering”.

6.3.2. Cell Line Maintenance

6.3.2.1. Jurkat (ATCC#TIB-152)

Propagation: RPMI 1640, 5\% FBS, 1\% P/S, 1\% L-Glu

Subculturing: Check cell concentration everyday and maintain cultures at a concentration between 1 x 10\textsuperscript{5} and 1 x 10\textsuperscript{6} viable cells/ml. Do not allow the cell concentration to exceed 3 x 10\textsuperscript{6} cells/ml.

6.3.2.2. Raw 264.7 (ATCC#TIB-71)
Propagation: RPMI 1640, 5% FBS, 1% P/S, 1% L-Glu

Subculturing: Subcultures are prepared by scraping. Dislodge cells from the plate substrate with a cell scraper; aspirate and add appropriate aliquots of the cell suspension into new culture plates. Passage daily. Do not passage over P34.

6.3.2.3. THP-1 (ATCC# TIB-202)

Propagation: RPMI 1640, 10% FBS, 1% P/S, 1% L-Glu

Subculturing: Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspend at 2-4 x 10^4 viable cells/ml. Subculture when cell concentration reaches 8 x 10^5 cells/ml. Do not allow the cell concentration to exceed 1 x 10^6 cells/ml.

6.3.2.4. M2-10B4 (ATCC # CRL-1972)

Propagation: RPMI 1640, 10% FBS, 1% P/S, 1% L-Glu

Subculturing:

1. Incubate PBS and medium in 37°C water bath for 15 minutes
2. Use a 10 ml pipette to remove the old medium
3. Use 5 ml warm PBS to wash the 25 cm² flask
4. Add 0.5 ml 0.25% trypsin/EDTA to the flask
5. Close the seal and gently turn the flask side to side till trypsin/EDTA covers the whole bottom area
6. Remove trypsin/EDTA and incubate the flask in 37°C incubator for 3-5 minutes until all cells detach
7. Wash the flask with 5 ml warm medium to suspend the cells, suck and blow for 4, 5 times
8. Make dilutions according to cell concentration and return the flask to incubator

6.3.2.5. NIH/3T3 (ATCC#CRL-1658)

Propagation: DMEM, 10% Calf Bovine Serum, 1% P/S, 1% L-Glu

Subculturing:

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.25% (w/v) trypsin - 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5-15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Add appropriate aliquots of the cell suspension to new culture vessels.

6. Incubate cultures at 37°C.

DO NOT ALLOW THE CELLS TO BECOME CONFLUENT! Subculture at least twice per week at 80% confluence or less.

Subcultivation Ratio: Inoculate 3 to 5 x 10^3 cells/cm^2

6.3.3. Labeling Experiments

6.3.3.1. Preparing Cell Lysates

1. Get cells and count them, wash once and resuspend in buffer to desired concentration
2. Sonicate the suspended cells for 3 seconds each time at 40 or 60 power, according to the volume, usually use smaller power for lower power because it will spill more with higher power. Repeat 10 times with 1 min break to make sure it cools down enough. Use ice bath. The solution should appear uniform yet not all clear at the end.

3. Transfer to centrifuge tubes and centrifuge at 100,000g for 45 minutes.

4. Resuspend the membrane pellet in buffer according to experimental need. I usually make it 1mg/ml-4mg/ml for THP-1 cells.

6.3.3.2. Western Blotting

A. Transfer protein to the PDVF membrane

Refer user’s manual for Bio-Rad Blotter.

B. Detection:

1. Remove blot from the transfer apparatus and block nonspecific sites with Blocking Reagent for 1hr at RT with shaking. If desired, block overnight at 2-8ºC without shaking. Briefly rinse with washing buffer.

2. Remove the Blocking Reagent and add the primary antibody working dilution. Incubate blot for 1 hour at RT with shaking or overnight at 2-8ºC without shaking.

3. Briefly rinse membrane in Wash Buffer two times.

4. Wash membrane by suspending it in Wash Buffer and agitating for ≥ 5 minutes. Replace. Wash Buffer at least 4-6 times. Increasing the Wash Buffer volume, the number of washes and wash duration may help minimize background signal.

5. Incubate blot with the HRP-conjugate working dilution for 1 hour at RT with shaking.
6. Repeat Steps 3 and 4 to remove nonbound HRP-conjugate.

Note: Membrane MUST be thoroughly washed after incubation with the HRP-conjugate.

7. Prepare the substrate working solution by mixing equal parts of Detection Reagents 1 and 2. Use 0.125 ml Working Solution per cm² of membrane.

Note: For best results prepare working solution immediately before use. The working solution is stable for up to 1 hour at RT.

8. Incubate blot with working solution for 1 minute at RT.

9. Picture the membrane immediately by Typhoon Trio, chemiluminescence mode, medium sensitivity, 100 pixels. Select only the area of the membrane. Save, export to bmp format and save as pdf. Wipe the scanner after use.

6.3.4. Transfection of NIH/3T3 Cell Line

6.3.4.1. Plasmid Preparation

1. 1 μl plasmid stock is added to 40 μl DH10B cell culture, mix well and transfer cells to cuvetts. Make sure no bubbles inside and shake the solution to the bottom. This should be done on ice. Move cuvetts, pre-warmed LB media, 1ml pipets and 12 ml culture tubes to the cold room.

2. Set the electroporator to: Bacteria, 2.49 kV; wipe dry the outside of cuvetts, place it in and hit pulse. Wait until the screen shows a number, which should be greater than 4. Add 1ml LB media immediately to it and mix, then transfer to culture tubes.

3. Incubate immediately at 37°C shaker for an hour. Pre-warm the LB/AMP plates for later use.
4. Label plates at the bottom, dip glass rods in pure ethanol and burn it to dry, wait until it cools.

5. Pipet 40 μl of LB cell stock to each plate: Test the rod on media plate first then spread the cells on media evenly. You may save the rest of the LB cell stock at 4°C for now. Incubate plates with media side on top at 37°C for a period of 14-18 hours.

6. Pick a single colony with pipet tips (sterile), drop the tip in 5 ml LB media in a culture tube, incubate at 37°C for 16-18 hours.

7. Preparation of glycerol stocks: Mixed 300 μl 50% glycerol solution and 600 μl saturated culture and snap freeze it. Store at -80°C for later use.

8. Centrifuge 3 ml saturated culture at 13.2krpm for 2 minutes at 4°C, remove media.

9. Add 300 μl buffer P1 and resuspend the cells, then add 300 μl of P2, flip the tube a few times, leave it for 1 minute and when flip again it should appear clear. Add 450 μl N3 to it and it should appear cloudy after it is flipped a few times. Centrifuged at 13.2 krpm for 20 minutes at 4°C.

10. Wash the vacuum kit and sit columns on it. Transfer the supernatant after centrifugation to the columns and turn on vacuum, break the vacuum by removing one of the plugs on other ports. Add 500 μl buffer PB, vacuum, followed by 750 μl buffer PE and vacuum. Spin the columns at 13.2 krpm for 2 minutes at room temperature and discard the solution.

11. Prepare Elution Buffer (EB) with 10mM Tris.HCl at pH 8.0. Add 40 μl EB to filter paper in the column, let it soak for 2 minutes and spin at 13.2 krpm for 1
minute, repeat this once more. Collect the 80 μl solution after centrifugation, which contains the plasmid.

6.3.4.2. Cell Transfection

For detailed information about lipofectamine 2000, refer “lipofectamine2000_man” file in the Manual folder included in the attached CD. Here a procedure is included on transfection of NIH/3T3 cell line with the pEBB-BAI1-GFP plasmid on a 12 well plate.

1. One day before transfection, 0.1 million cells are plated in each well with 1ml complete growth media without antibiotics. Cells should be 90-95% confluent at the time of transfection.

2. For each well:
   a. Dilute 10 μl DNA (~0.2 μg/μl) with 90 μl Opti-MEM I and mix.
   b. Mix lipofectamine 2000 gently before use, then add 4 μl of it to 96 μl Opti-MEM I, mix and incubate at room temperature for 5 minutes. (Proceed to step c within 25 minutes)
   c. Combine the diluted DNA with the diluted lipofectamine 2000 (100 μl+100 μl), mix gently and incubate at room temperature for 20 minutes.

3. Add 200 μl of complexes to each well, mix gently by rocking the plate back and forth.

4. Incubate cells at 37°C in a CO₂ incubator for 24 hours prior to testing for transgene expression.

6.3.5. PMA Treatment on THP-1 Cell Line

Solid PMA (Sigma) is dissolved in sterile filtered DMSO to give a 1mg/ml stock. Store in 20 μl aliquots at -20°C, in dark.
1. THP-1 cells are counted and adjusted to 0.5 million/ml, add PMA stock to give a 100 ng/ml final concentration. Incubate for 48 or 72 hours in petri dish (10x10 cm, 20 ml cells per dish)

2. Cells become adherent. Remove PMA containing media and rinse twice with DPBS, added 15 ml fresh media and incubated for 24hrs.

3. Cells are scraped or trypsin digested off the plates, counted and centrifuged (150xg, 5 minutes) for later experiments.

Media: RPMI 1640, 10%FBS, 1% L-Glu and 1% P/S. THP-1 cells are lower than P30.

6.3.6. Sample Preparation for FACS

1. For adherent cells, digest them with trypsin and wash with buffer, resuspend at 0.5-1.0 million/ml concentration in 200 μl buffer and add 200 μl 4% HCHO, mix gently and filter if needed.

2. Let it fix for at least 20 minutes in 4°C.

3. Measure by FACS. Keep sample in dark all times to avoid possible photo bleaching.

6.4. References for Chapter 6

BIBLIOGRAPHY


