SMALL MOLECULES FOR THE SELECTIVE RECOGNITION OF

BIOMEMBRANE COMPONENTS

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This dissertation describes the design and evaluation of small molecules that interact specifically with certain components of the cell plasma membrane surface and interior. A series of Zn\(^{2+}\)-DPA coordination compounds has been developed that can report the presence of phosphatidylserine in the outer leaflet of a cell membrane in the same manner as the protein Annexin V, but with no need for calcium. These compounds include fluorescent probes suitable for flow cytometry and fluorescence microscopy, as well as a biotinylated sensor for phosphatidylserine that can be adapted to sensing applications for which suitable streptavidin conjugates are available. Direct conjugation of these phosphatidylserine-binding compounds to quantum dots resulted in a sensor useful for prolonged exposure imaging applications without bleaching of the fluorophore. A second series of amphiphilic compounds designed to partition into the bilayer hydrocarbon region and interact with membrane proteins is also presented. These molecules include derivatives of the lipid phosphatidylcholine, and all have either thymine or urea functionalities that likely embed in the bilayer and form hydrogen bonds.
to targeted transmembrane protein residues. All compounds presented in this work have either immediate applications in diagnostic imaging or the potential to help us understand more about the process of molecular recognition in a cell membrane interior.
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professional development by all those scientists fortunate enough to have spent time in
the Smith Group Research Laboratories.
ABBREVIATIONS

Å  Angstrom (10^{-10} m)
ATP  adenosine triphosphate
°C  degrees Celsius
CF  carboxyfluorescein
CHCl₃  chloroform
δ  chemical shift
DAGK  diacylglycerol kinase
DPA  2, 2’-dipicolylamine
DHPC  1,2-dihexanoyl-sn-glycero-3-phosphocholine
DHPS  1,2-dihexanoyl-sn-glycero-3-phosphoserine
DLS  dynamic light scattering
FITC  fluorescein isothiocyanate
g  gram
µL  microliter (10^{-6} L)
M  molar (moles per liter)
mg  milligram
NADH  nicotinamide adenine dinucleotide (reduced)
NBD  7-nitrobenz-2-oxa-1,3-diazole-4-yl
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>PK</td>
<td>pyruvate kinase</td>
</tr>
<tr>
<td>POPA</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-phosphatidic acid</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine</td>
</tr>
<tr>
<td>POPG</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-phosphoglycerol</td>
</tr>
<tr>
<td>POPS</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-phospho-L-serine</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PSS</td>
<td>phosphatidylserine sensor</td>
</tr>
<tr>
<td>SM</td>
<td>sphingomyelin</td>
</tr>
<tr>
<td>TES</td>
<td>N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>7AAD</td>
<td>7-Aminoactinomycin D</td>
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CHAPTER 1
MEMBRANE STRUCTURE AND CHEMICAL INTERACTIONS IMPORTANT FOR MEMBRANE FUNCTION

1.1 Introduction

Our understanding of the plasma membrane of eukaryotic cells has grown considerably since the fluid mosaic model was introduced by Singer and Nicholson.\(^1\) Elegant experiments and sophisticated theoretical calculations have resolved the picture of a cell membrane from a coarse polar-surface-apolar-interior view to the more detailed structure described today.\(^2\) We now know that the cell membrane is a dynamic environment rich in chemical functionality\(^3\) (Figure 1.1). Though the molecular contacts that take place in the bilayer interior and on the bilayer surface involve a panoply of chemical interactions, we are beginning to understand the dominant role of certain interactions in the various environments of the membrane. At the surface of the membrane, for example, electrostatic interactions play an important role in properly orienting proteins for membrane binding.\(^4\) In the membrane interfacial region, ion-diople interactions are common stabilizing forces,\(^5\) and van der Waals forces and hydrogen bonding contribute significantly to molecular interactions in the hydrocarbon interior.\(^6\) The common theme in each case is the non-covalent, supramolecular nature of the chemical interactions that dominate molecular recognition in each area of the membrane.
The principle non-covalent interactions that frequently occur in the plasma membrane environment are electrostatic and cation-π interactions, hydrogen bonding, and van der Waals forces. These chemical interactions provide an important framework for attempting to rationalize the potential effects of point mutations in transmembrane or membrane-binding proteins, as subtle changes in chemical functionality in certain membrane environments can lead to remarkable changes in the chemical behavior of the protein.

Figure 1.1 – Noncovalent interactions play critical roles in maintaining cell membrane structure and facilitating membrane function. The membrane components, phospholipids, proteins, cholesterol, and inorganic ions, all interact through hydrogen bonding, van der Waals contacts, electrostatic attractions, and cation-π interactions to create a heterogeneous environment with rich chemical properties.

From a chemist’s perspective, the cell membrane is a structure with three distinct environments, the apolar hydrocarbon interior of the bilayer, the midpolar interfacial region containing both the glycerol backbone and the acyl portion of the fatty acid tails, and the polar phospholipid headgroup region (Figure 1.2). The hydrocarbon interior is the primary barrier for movement of charged species across the membrane. The low dielectric constant of the hydrocarbon interior prevents most charged or polar species
from penetrating the bilayer beyond the acyl region, and most proteins that span the bilayer have a sequence of apolar residues to match the hydrocarbon region. One important consequence of such a low dielectric environment, however, is the highly favorable driving force for interaction created when mutations introduce polar residues that can hydrogen bond or form salt bridges into the transmembrane region of proteins.\textsuperscript{7}

![Diagram of membrane regions](image)

**Figure 1.2** – The plasma membrane of eukaryotic cells has three chemically distinct regions in which proteins are frequently found, the polar headgroup region (A), the midpolar interfacial region (B), and the apolar hydrocarbon region (C).

The more polar interfacial and headgroup regions of the membrane, on the other hand, are rich in chemical functionality and provide ample opportunity for protein-lipid interactions by presenting ammonium and carboxylate residues found in the headgroups of the common phospholipids, along with carbonyl groups located in the acyl region of the phospholipid backbone.

Eukaryotic cell membranes have four primary phospholipid components, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and sphingomyelin (SM) (Figure 1.3), distributed asymmetrically between the inner and the outer leaflets of the membrane.\textsuperscript{8-9} The chemical functionality presented by these, along
with other minor phospholipids, makes specific protein-lipid interactions possible, all mediated largely by electrostatics, hydrogen bonding, van der Waals forces, and cation-π interactions. Previous reports have summarized these forces in the context of small molecule interactions in solution,¹⁰ and their importance in protein-membrane and transmembrane protein-protein interactions will be considered further in the research described here.

**Figure 1.3** - Structure of the common cell membrane phospholipids phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), and phosphatidylserine (PS).
1.2 Key Forces Involved in Membrane Structure and Function

1.2.1 Electrostatic Forces

The extracellular domains of many transmembrane proteins display charged amino acids on the membrane surface which frequently serve as anchors to position the proteins correctly for ligand binding and other biochemical activity. Attractive interactions between oppositely charged proteins and a cell membrane or between transmembrane proteins and their binding partners are often responsible for initiating protein-membrane or protein-protein binding. This important initial interaction often positions the protein to subsequently form additional stabilizing contacts with specific functional groups at the membrane surface.

The human growth hormone receptor (hGHR) is a single-pass transmembrane protein with an extracellular ligand binding domain projecting from the membrane surface. Human growth hormone is a peptide that binds to hGHR at a rate approximately 10,000 fold slower than the diffusion limit of the hormone, but approximately 1,000 times faster than expected if hormone-hGHR binding required the hormone to collide with the receptor in the correct orientation for binding.\textsuperscript{11} This enhanced value for the association rate, $k_{\text{on}}$, arises from electrostatic attractions between four key Arg residues on the hormone and negatively charged groups on the hGHR that causes the hormone to approach the hGHR in the proper orientation for binding.\textsuperscript{12} This charge-charge interaction specifically orients the hormone for subsequent formation of stabilizing contacts that form in the ligand binding site, to be discussed in detail below. This
electrostatic interaction is estimated to contribute to the enhancement of $k_{on}$ by
approximately a factor of 20.

Similar electrostatic driving forces are observed in the binding of phospholipid
membranes by certain phospholipases. For example, the human group IIa secreted
phospholipase A$_2$ (PLA$_2$), which preferentially binds anionic membranes, has an
association constant, $k_{on}$, for membrane binding 1,000-10,000 fold greater than random
diffusion would predict for a specific protein-ligand binding event. The
importance of electrostatics to this enhancement were revealed in charge-reversal mutants
of PLA$_2$, where the cationic residues surrounding the PLA$_2$ active site were mutated to
anionic residues. Such mutants exhibited a decline in $k_{on}$ of greater than ten fold. An
even more dramatic illustration of the power of electrostatic interactions is binding of the
protein Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) to anionic
phospholipid membranes, particularly those enriched in the anionic lipid
phosphatidylinositol-4,5-bisphosphate (PIP$_2$). The effector domain of MARCKS,
residues 151-175, contains 13 basic residues, making MARCKS an ideal binding partner
for anionic membranes. The interaction between MARCKS and anionic membranes is in
fact so favorable that the rate of association, $k_{on}$, has been approximated as diffusion
limited based on experiments using truncated versions of the protein with an intact
effector domain.

The formation of weakly bound protein-membrane or protein-protein complexes
by electrostatic forces is frequently followed by formation of specific contacts between
the interacting proteins or between protein residues and chemical functionality at the
membrane. These interactions stabilize the complex normally by decreasing the rate of
dissociation, $k_{\text{off}}$, resulting in a tightly-bound protein-protein or protein-membrane complex. The hGHR-growth hormone interaction discussed earlier provides an illustrative example. Upon binding of growth hormone to hGHR, 31 residues are buried in the hormone-receptor interface, yet approximately 85% of the total binding energy can be attributed to only eight key residues.

MARCKS-membrane binding also illustrates how important the post-binding formation of specific contacts can be to protein-membrane complex stability and protein activity. Upon membrane binding, five Phe residues in the effector domain of MARCKS partition deeply into the bilayer, pulling the MARCKS protein down onto the membrane-water interface and forcing the protein backbone into the membrane interfacial region. In the Phe→Ala mutant, where all five Phe residues have been mutated to Ala, the protein continues to bind the membrane, but remains separated from the surface by a distance of approximately 10 Å, held in place purely by long-range Coulombic interactions. The simultaneous contribution of electrostatic, hydrophobic, and van der Waals forces that results in MARCKS binding and partitioning into the membrane interfacial region are all clearly important in MARCKS-membrane interaction, and similarly designed systems may also need to exhibit such a simultaneous dependency on multiple forces in order to achieve tight binding to the membrane.

The protein and the plasma membrane do not necessarily need to be of opposite charge for electrostatics to play a key role in membrane binding. Often a protein and membrane can form a tightly-bound complex via a bridging metal ion, especially $\text{Ca}^{2+}$. The C2 domain is a phospholipid binding domain originally observed in protein kinase C and commonly found in a number of phospholipases. This domain binds to anionic
membranes through a bridging Ca\textsuperscript{2+} in a cooperative manner,\textsuperscript{20} where neither the protein nor the membrane have a strong affinity for Ca\textsuperscript{2+}, but when all three components come together into a three-component-assembly, a stable protein-Ca\textsuperscript{2+}-membrane complex is formed. The cooperativity of membrane binding by the C2 domain of synaptotagmin I has been described, and two Ca\textsuperscript{2+} ions are known to be bound by the protein in the protein-ion-lipid assembly. The first Ca\textsuperscript{2+} is bound tightly, while the second occupies a binding site of weaker Ca\textsuperscript{2+} affinity. A phosphoryl oxygen of a phospholipid headgroup is required for tight Ca\textsuperscript{2+} binding and complex formation. Observations of the C2 domain in protein kinase C α (PKCα) have uncovered similar binding mechanisms, where bridging Ca\textsuperscript{2+} ions anchor the phospholipid,\textsuperscript{21} especially PS, into the binding site of the C2 domain, which simultaneously increases the Ca\textsuperscript{2+} affinity of the complex. Importantly, the presence of a bridging Ca\textsuperscript{2+}, and not simply a center of cationic charge, is absolutely required for binding. PKC mutants with cationic residues introduced into the C2 domain of PKCα where Ca\textsuperscript{2+} would normally be found were shown to be unable to bind anionic membranes.\textsuperscript{22} Identical observations have also been made using the PS-binding protein Annexin V,\textsuperscript{23} which requires at least two Ca\textsuperscript{2+} ions to simultaneously coordinate the carboxylate and the phosphate of PS. These Ca\textsuperscript{2+} ions form the bridge that links Annexin V and PS in a tightly-bound three-component assembly, a membrane binding mechanism that is highly conserved among the Annexin family of proteins.

1.2.2 Cation-π Interactions

Sequence analysis of transmembrane proteins reveals that the amino acids in the membrane-spanning portion of the protein do not occur randomly. The hydrocarbon-
spanning sequence is found to be rich in Leu, while the amino acids of the interfacial region are rich in Trp and Tyr. These aromatic amino acids, especially Trp, provide a significant stabilizing force for membrane protein positioning in the bilayer by forming cation-π interactions with the ammonium groups of the membrane phospholipids. The stabilizing effects of cation-π interactions are well known in biological systems, and their importance in protein-membrane and transmembrane protein-protein interaction is just beginning to be understood. The ammonium-Trp interaction is favorable by 2-4 kcal/mole in some systems, and in the supramolecular context of protein-membrane and protein-protein complex formation, such a stabilizing effect can make a substantial contribution to overall complex stability.

Both computational and experimental data confirm that model peptides containing Trp interact favorably with the headgroup of phosphatidylcholine by forming both cation-π interactions between the indole ring of Trp and the quaternary ammonium of PC, as well as hydrogen bonds between the indole N-H and the phosphate oxygens. These same experiments discovered a role for the phenolic side chain of Tyr as well. Here, the formation of a hydrogen bond between the PC phosphate and the Tyr hydroxyl was more favorable than the potential cation-π interaction with the PC quaternary ammonium.

In the hGHR, a transmembrane protein described earlier, the extracellular domain is stabilized by an extended network of cation-π interactions. Crystal structures of hGHR revealed a series of amino acid side chains packed together into an overlapping arrangement where cation-π interactions, hydrogen bonds, and a salt bridge all play a role in stabilizing the protein structure. The geometry of the packed side chains seemingly makes it possible for key residues to participate simultaneously in two or more stabilizing
interactions. For example, Arg213, which forms a salt bridge with Glu175, also seems positioned to interact favorably with the aromatic ring of Phe225 and/or Tyr222 in a cation-π interaction. Other obvious cation-π interactions in this structure are noted, including that between Arg211 and Trp186. Importantly, the stabilizing interactions in hGHR are formed between non-consecutive amino acids in the primary structure of the protein, indicating that these interactions primarily stabilize the secondary and tertiary structure of hGHR, a feature that underscores just how critical these forces can be for maintaining proper structural and functional protein integrity.

A clear illustration of cation-π interactions at work in protein-phospholipid binding is provided by the anti-PC antibody McPC603, which binds the headgroup of PC. Again, cation-π interactions work in concert with other forces to facilitate formation of a tightly-bound protein-phospholipid complex. The quaternary ammonium of PC makes contact with Trp107 of the heavy chain of McPC603, interacting with the partially negative ring face of the indole. Additionally, the phosphate group of PC also participates in two important interactions with McPC603, one to Tyr33 of the McPC603 heavy chain, and one to Tyr100 of the light chain. Interestingly, these Tyr rings associate with the phosphate not through their hydroxyl groups, but through contacts with the protons of their partially positive ring edge. These phosphate-ring edge contacts contribute a combined favorable interaction energy of approximately 1.25 kcal/mole. The McPC603 protein itself also exhibits an important cation-π interaction not directly involved in phospholipid binding. Arg52 and Trp107, both from the McPC603 heavy chain, form a cation-π interaction resulting in 23 Å² of combined contact area. This interaction turns out to be critical for PC binding, as all affinity for PC
was lost when Arg52 was mutated to residues incapable of forming cation-π interactions. The nature of the Arg-Trp cation-π interaction was examined further in model peptides using computational techniques, and found to be more favorable in the environment of a membrane interfacial region than when free in solution. The lifetime of an Arg-Trp interaction was longer at a membrane surface than in bulk aqueous solution, and the stability of the Arg-Trp interaction was found to be greater than that of the Arg-Phe interaction in nearly every instance.

The influence of cation-π interactions on small molecules designed to bind or insert into the membrane was illustrated by a recent report of synthetic ion channels found to depend on cation-π stabilizing interactions for maximum activity. This work demonstrated clearly how cation-π interactions at a phospholipid membrane surface can exert a dramatic effect on the activity of a designed molecule intended to perform a desired function at or in a membrane bilayer.

1.2.3 van der Waals Forces

The chemical interactions in the interior of the bilayer also illustrate important principles of supramolecular chemistry necessary for proper membrane and membrane protein function. The apolar hydrocarbon region of the bilayer consists of phospholipid acyl chains of varying lengths and degrees of unsaturation. In some cases, these lipids pack together to form discrete domains in the membrane. These “lipid rafts,” which are commonly rich in SM and cholesterol, result from phospholipids and cholesterol coming together in a way that maximizes van der Waals contacts. These contacts are particularly significant in the case of cholesterol interactions with fully saturated
hydrocarbon tails. The magnitude of van der Waals forces in SM-cholesterol interactions was found to be even greater than the effects of hydrogen bonding between the cholesterol hydroxyl and the potential hydrogen bonding functionalities of SM.\textsuperscript{38} This illustrates the importance of molecular packing arrangements in the membrane interior in systems designed to populate the hydrocarbon region of the bilayer.

Another consequence of the variability in acyl chain length among the membrane phospholipids is the phenomenon of hydrophobic matching. This ordering of phospholipids in the membrane occurs when transmembrane proteins of varying lengths cause localized thickening and thinning of the membrane bilayer when the protein and the phospholipids pack together in an orientation that maximizes van der Waals contacts. Perhaps the most well-studied example of such a process is dimerization of the protein gramicidin D (gD),\textsuperscript{39} which self-associates in a membrane to form a bilayer-spanning channel. gD is a mixture of six 15-amino acid, \textit{N}-formyl helical peptides that span a length approximately equal to one leaflet of a membrane bilayer. When two gD monomers, one in each bilayer leaflet, contact each other as they diffuse about their respective monolayers, they dimerize to form a transmembrane channel. The phospholipids in each leaflet then pack around the gD dimer so that maximum hydrophobic matching occurs, therefore maximizing van der Waals contacts between the proteins and the phospholipids, and also between the lipids themselves. Hydrophobic matching can result in a local thickening or thinning of the membrane, depending on the membrane composition. For instance, membranes consisting of DLPC (1,2-dilauroyl-\textit{sn}-glycero-3-phosphocholine) or DMPC (1,2-dimyristoly-\textit{sn}-glycero-3-phosphocholine) are approximately 30.8 and 35.5 Å thick, respectively. However, the addition of gD at a
lipid:peptide ratio of 10:1 results in a thickness adjustment of each membrane to approximately 32 Å, consistent with a lipid reorganization in the bilayer intended to maximize van der Waals contacts and hydrophobic matching.\textsuperscript{39}

Transmembrane protein-protein interactions can also be affected by van der Waals forces, as illustrated by dimerization of the transmembrane protein glycophorin A (gpA).\textsuperscript{40} On first inspection, gpA does not appear structurally prone to self-association. It lacks polar residues for hydrogen bonding in its transmembrane domain, which is rich in Gly, Val, and Ile residues. However, these small hydrophobic residues form a dimerization interface that allows two gpA monomers to associate tightly into a dimer stabilized by van der Waals forces. Direct interhelical packing occurs between Gly residues 79 and 83 of each helix, consistent with transmembrane interhelical interactions mediated by the commonly observed GxxxG motif.\textsuperscript{41-42} In this case, the very small side chain of Gly, a single proton, occurs at the $i$ and $i+4$ positions, aligning them correctly for formation of a dimerization interface that can drive helix-helix binding by van der Waals forces. Additional interactions between Ile76 and Gly 79 and between Val80 and Gly83 of gpA further lock the two helices together into the dimer, which is stabilized by the total van der Waals forces that result from the close interhelical packing.

1.2.4 Hydrogen Bonding

The chemical functionalities commonly encountered on a membrane surface, including hydroxyls, carboxylates, phosphates, and ammoniums, are often key points of attachment for proteins that bind a phospholipid. Like all other noncovalent forces involved in protein-membrane binding, hydrogen bonding is usually just one force
contributing to the overall energy of binding. However, the importance of hydrogen bonding in protein-membrane and transmembrane protein-protein interaction can hardly be overstated, both on the membrane surface and in the membrane interior.

Using small model peptides, generalizable hydrogen bonding interactions between amino acid side chains and the phospholipid headgroups have been described. In particular, the interaction between the Trp indole N-H proton and the phosphate of a phospholipid has been investigated. Trp residues are often found in the portion of transmembrane proteins that localize to the membrane interfacial region. The combination of cation-\(\pi\) and hydrogen bonding interactions made possible by the Trp indole ring may explain the frequency with which Trp residues are observed at the interfacial region, where they likely serve as “membrane anchors” for the transmembrane proteins.

Hydrogen bonding in the bilayer hydrocarbon region is an important noncovalent interaction governing biological events in both the normal and a variety of disease states. Gramicidin A (gA), a 15 amino acid, \(N\)-formyl component of the gD mixture described earlier, is a naturally occurring antibacterial protein that inserts into phospholipid membrane bilayers and then dimerizes to form channels permeable to metal cations. The gA dimer forms when two gA monomers associate in a head-to-head fashion in the membrane, followed by formation of six stabilizing hydrogen bonds between the \(N\)-formyl ends of each monomer. These hydrogen bonds form between backbone N-H protons and backbone carbonyl oxygens at residues \(O^1\cdots N^5\cdot H\), \(O^3\cdot N^2\cdot H\), and \(O^5\cdots N^1\cdot H\). Two sets of hydrogen bonds form at these locations, one set from each gA monomer. All six hydrogen bonds are necessary for effective channel formation. This was
demonstrated by the decrease in channel function and channel lifetime that resulted from replacement of just one of the indicated amide bonds by an ester bond, eliminating a single hydrogen bond donor.\textsuperscript{44}

Transmembrane proteins that completely span the bilayer, unlike gA, normally have an apolar amino acid sequence spanning the hydrocarbon interior of the bilayer. The low dielectric constant of this environment makes the hydrocarbon interior a high energy, unfavorable site for polar amino acids. Mutations that introduce these residues, particularly those that can participate in hydrogen bonding, into the hydrocarbon region often lead to unfavorable phenotypes.\textsuperscript{7, 45} Many transmembrane proteins are receptors for hormones that exert their effect by inducing receptor dimerization or conformational change. In instances where polar amino acids are introduced into the transmembrane region of these receptor proteins, the stability imparted by hydrogen bonding is often a sufficient driving force for ligand-independent receptor dimerization, a situation that can lead to numerous cellular malfunctions.

The chemical properties of common functional groups can change dramatically between the aqueous extracellular fluid and the apolar hydrocarbon interior of a bilayer. A carboxylic acid, for example, can experience a shift in pKa from 4.5-5 in water to as high as 8.5-9 in a bilayer membrane.\textsuperscript{46} The energetic cost of burying these polar residues in the apolar bilayer interior is substantial. Amide bonds, for example, have a free energy of transfer from an aqueous environment to an apolar environment of approximately +6 kcal/mole, but the required energy is decreased by about an order of magnitude, to around +0.6 kcal/mole, when the amide bond participates in hydrogen bonding.\textsuperscript{47} This illustrates how favorable hydrogen bonding in the membrane interior can
be, and underscores the importance of such interactions that arise out of mutations in a transmembrane peptide sequence.

An important example of mutations that introduce a hydrogen bonding residue into a transmembrane protein sequence are the common mutations found in the transmembrane sequence of certain receptor tyrosine kinases. A Val664Glu mutation in the transmembrane domain of the Neu receptor tyrosine kinase causes ligand-independent receptor dimerization and upregulation of constitutive kinase activity. Two hypotheses for the mechanism of this upregulation have been suggested, both involving hydrogen bonding in the bilayer interior. In the first, the protonated carboxyl side chain of Glu664 forms a cyclic hydrogen bonded structure that stabilizes the Neu dimer by two hydrogen bonds between the side chains of Glu664 in each of two neighboring helices. In the second, the OH of the protonated Glu664 forms a hydrogen bond to the backbone carbonyl of Ala661 of a neighboring Neu monomer, again allowing the dimer to be stabilized by two hydrogen bonds, but without the cyclic structure proposed in the first model. The ligand-independent receptor dimerization brought about by the Val664Glu mutation in the Neu receptor leads to a number of downstream cell signaling events, including unchecked cell growth and possible roles in cancer.

A residue capable of hydrogen bonding that appears in a transmembrane sequence has at least three different opportunities to form hydrogen bonds. It can self associate, forming hydrogen bonds with the side chain of the same residue on a neighboring helix, it can form hydrogen bonds to another polar residue, or it can hydrogen bond to the carbonyl of the protein backbone. Backbone C=O participation in hydrogen bonding with polar residues is a commonly observed stabilizing interaction in transmembrane
protein sequences, but carbonyl hydrogen bonding has also been described as an important feature in a much less common interaction, that of Cα-H···O hydrogen bonding. With an approximate pKa of 18-20, the Cα proton falls far short of those of the more commonly observed hydrogen bond donors in terms of acidity, but Cα hydrogen bonds have nevertheless been suggested to help stabilize helix-helix dimers, especially those with the GxxxG motif, including gpA, where the helices can form especially close contacts. Though computational estimates of Cα-H···O bond strength in the gas phase value the interaction at as much as 2.5-3 kcal/mole, experimental data from actual lipid bilayer measurements of Cα-H···O bond strength value the interaction at only 0.88 kcal/mole. This means that if these hydrogen bonds are to contribute to helix-helix interactions in any meaningful way, several of such interactions will likely be required simultaneously.

1.3 Summary and Directions for Research

The complex environment of the cell membrane makes development of compounds to selectively interact with any membrane component a daunting challenge. The opportunity to participate in hydrogen bonding, coulombic attractions, cation-π interactions, and van der Waals contacts means that designing compounds to associate with a given membrane protein, carbohydrate, or phospholipid, in preference to all the others, must be done by carefully considering the environment of the target and the interactions in which it participates. The research described in the following chapters addresses the problem of molecular recognition at a membrane surface by considering how nature applies the principles of supramolecular chemistry to specific recognition of
selected phospholipids and protein components in the environment of a bilayer membrane. By carefully designing molecules that can form the same chemical interactions with a membrane component as its natural binding partner, small molecules have been developed that can specifically associate with a targeted component of the bilayer membrane in the presence of all the others. In each case described, specific recognition has only been achieved by engineering a combination of weak interactions to take place simultaneously between the designed molecules and their membrane binding partner(s). This research is a clear illustration of the power of weak interactions in chemistry and biology. The importance of considering the aggregate effects of the environment in which a chemical process takes place, as well as the role of subtle changes to molecular structure are identified here in the context of molecular recognition of membrane phospholipids and selected membrane proteins.
2.1 Introduction

The four principle phospholipid components of a eukaryotic cell membrane\textsuperscript{8}, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) are distributed between the two monolayers of the membrane in an asymmetrical fashion. The choline-containing lipids, PC and SM, largely populate the extracellular leaflet, while the aminophospholipids, PE and especially PS, are restricted primarily to the inner membrane leaflet\textsuperscript{9,55-58} (Figure 2.1). This membrane asymmetry has been known for some time, and there is a consensus that it is maintained by the concerted action of a family of translocase enzymes. Efforts to elucidate the structure and mechanism of these transport proteins are ongoing and are described elsewhere.\textsuperscript{8,59-60} Many recent attempts have been made to develop sensing strategies that report a breakdown in this membrane asymmetry, a signature event indicating that cells have entered the early-to-middle stages of apoptosis.\textsuperscript{61-62}

Apoptosis, or the sequence of cellular events collectively known as “programmed cell death,” is an important process whereby cells are intentionally marked for clearance from the body. Apoptosis is a central process in developmental biology and also in many
types of diseases. For example, selective induction of apoptosis in cancerous tissue is an attractive chemotherapeutic strategy, and detection of apoptosis is therefore a key step in the drug development process. Various strategies for detecting apoptosis have been reported, including monitoring of intracellular caspase activity, observing nucleic acid fragmentation, and detection of membrane permeabilization. These assays are employed as diagnostic tools for identifying apoptosis, but each has limitations that render it imperfect in certain situations.

![Figure 2.1 - Distribution of the common membrane phospholipids between the inner and outer monolayers of the membrane bilayer. Adapted from Reference 61.](image)

Loss of the phospholipid asymmetry inherent to healthy animal cell membranes is a hallmark of apoptosis, regardless of the initiating stimulus. During the early to middle stages of apoptosis, the PS normally found exclusively on the inner membrane monolayer becomes scrambled between the two membrane leaflets. PS is the only anionic phospholipid component of the plasma membrane that is present in appreciable levels,
and externalization of PS results in a net buildup of anionic charge on the membrane surface. There is abundant evidence suggesting that PS externalization is a contributing factor to the recognition of dead and dying cells by macrophages. The externalized PS can be detected on the cell surface using indicator-labeled reagents that preferentially bind the PS headgroup. PS externalization precedes the upregulation of protease activity in the cytosol, and occurs long before membrane permeabilization begins. A particularly attractive feature of this cell surface assay is that it avoids the complications of other assays that require access to the cytosol. Furthermore, this strategy for PS recognition makes it possible to consider applications for site-specific in vivo imaging of apoptotic tissue that would be useful in the treatment of various diseases such as cancer and cardiovascular disease.

2.2 Annexin V as a PS-Detection Reagent

The annexins are a group of proteins with presently undetermined function, all of which bind anionic phospholipids in a Ca\(^{2+}\)-dependent manner. One member of the family, Annexin V (Anx V), binds PS with high selectivity and high affinity, making it well suited for detection of apoptosis. A variety of fluorophore-labeled versions of Anx V are now commercially available, and detection of cell-surface PS by this technique has become a standard protocol in cell biology research. The basic structural and PS-binding features of Anx V have been described in previous reports. The simplicity of the structural and biochemical features of Anx V that confer its PS-recognition capabilities, as well as the limitations of Anx V, make development of non-protein PS recognition compounds a promising research topic.
A number of Anx V crystal structures have been solved, and the $\text{Ca}^{2+}$ coordination and PS binding geometry is well characterized. Anx V is a 36 KDa, single stranded protein. Its structure contains four domains, each consisting of five alpha-helical regions, and a short N-terminal sequence, making it unique among annexins, most of which have more elaborate N-terminal residues which are thought to confer a specific function. While the challenge of achieving a crystal structure of a membrane-bound Anx V has yet to be overcome, crystals of Anx V bound to glycerophosphoserine have been described. This initial report indicated a potential binding site for PS in domain 3 of Anx V, but this site was later found to contribute only weakly, if at all, to PS binding, while sites in domains 1 and 2 were required for PS binding.

Based on the available crystal structures, three potential sites for $\text{Ca}^{2+}$ coordination have been identified in each domain, and one in each domain has been found to exhibit higher $\text{Ca}^{2+}$ affinity than the others. In the Anx V crystal structure incorporating a bound glycerophosphoserine, two $\text{Ca}^{2+}$ ions separated by 8.7 - 8.8 Å were coordinated to the PS headgroup, one bound to the phosphoryl oxyanion and the other to the carboxylate (Figure 2.2). Docking studies later revealed a PS binding consensus sequence in domain 1 of Anx V, $\text{Arg}^{25}$-$\text{X}_3$-$\text{Lys}^{29}$ . . . $\text{Arg}^{63}$-$\text{X}_4$-$\text{Asp}^{68}$-$\text{X}_2$-$\text{Ser}^{71}$-$\text{Glu}^{72}$, which was subsequently shown to be present in either domain 1 or 2, or both, in all known annexins. No other phospholipid has the same charge separation and geometrical orientation as PS, which suggests that the Anx V protein has evolved specifically to perform PS-binding functions.

As previously mentioned, appearance of PS on a cell surface is a general indicator of apoptosis, and binding of dye-labeled Anx V to cell membranes enriched in PS has
become a widely used technique for detecting apoptosis using flow cytometry\textsuperscript{33, 96} and fluorescence microscopy.\textsuperscript{74}  A number of Anx V conjugates bearing various reporter elements have been prepared and used for detection of apoptosis, and methodology for preparing customized Anx V conjugates has been described.\textsuperscript{97}  Josephson and coworkers have reported that Anx V retains its PS binding capabilities as long as the extent of modification does not exceed 1.6 modifications per mole of protein. The applications for which Anx V conjugates have been prepared are diverse, and include: FITC-labeled\textsuperscript{73, 96} and Phycoerythrin-labeled\textsuperscript{98-99} Anx V for flow cytometry and fluorescence-activated cell sorting (FACS), Cy5.5 conjugated Anx V for near-infrared (NIR) optical imaging,\textsuperscript{100-102} Anx V-\textsuperscript{18}F conjugates for positron emission tomography (PET),\textsuperscript{103-105} Anx V-Tc-99m conjugates for radioimaging,\textsuperscript{106-108} biotinylated Anx V for use with labeled streptavidin conjugates in a variety of applications,\textsuperscript{109-111} and metal and nanoparticle Anx V conjugates for cell separation and magnetic resonance imaging.\textsuperscript{112-113}

Even though Anx V derivatives are widely used for PS-sensing applications and apoptosis detection, Anx V is not without limitations. For instance, the unfunctionalized protein has a mass of approximately 36 KDa, which restricts its use to those applications where a PS sensor of this size can be accommodated. Furthermore, Anx V-PS binding requires millimolar levels of Ca\textsuperscript{2+} in order to produce the nanomolar dissociation constants that make using the protein desirable.\textsuperscript{76-78}  This level of Ca\textsuperscript{2+} may be problematic in situations where other processes are to be monitored simultaneously. Additionally, animal cells frequently have integral membrane phospholipid transport proteins, called “scramblases,” that can move phospholipids nonspecifically between the two membrane monolayers. These scramblases are activated by micromolar Ca\textsuperscript{2+}
levels,\textsuperscript{60} well below that necessary for Anx V-PS binding. This makes it necessary to consider the possibility that false positives may occur when using Anx V to detect apoptosis. Anx V-PS binding kinetics are also slower than what is needed to carry out certain kinetic assays. Complete membrane binding by Anx V often requires incubation periods of up to one hour,\textsuperscript{114} making kinetic assays difficult. The biochemical stability of Anx V has been discussed in previous reports, particularly noting that Anx V is susceptible to N-terminal proteolytic degradation.\textsuperscript{115-116} The aggregate of these limitations suggests that non-protein, small molecule sensors for PS would have multifarious applications.

2.3 Small Molecule PS Sensors and Mimics of Annexin V

2.3.1 Cationic Dyes that Sense Anionic Membranes

Sensing strategies for apoptotic cell membranes have employed small molecules, surface-functionalized nanoparticles, and supramolecular assemblies. The idea that small molecules can behave similarly to Anx V and signal changes in cell membrane properties is supported by use of the fluorescent dyes MC 540\textsuperscript{117} and FM 1-43,\textsuperscript{118} which have previously been used to detect apoptosis. The binding of MC 540 to membranes is sensitive to membrane composition, and binding increases when cells become apoptotic.\textsuperscript{61,119} However, there are significant disadvantages to use of MC 540. The signal difference between normal cells and apoptotic cells is only about five fold (it is up to 100 fold with Annexin V-FITC); furthermore, the dye is phototoxic. Likewise, FM 1-43 is a cationic dye that binds more tightly to membranes that are enriched in anionic phospholipids than those of normal healthy cells, but gives a signal difference between
normal and apoptotic cells of only 6-10 fold. Another disadvantage of FM 1-43 is its broad emission (515-595 nm), which makes it difficult to use a second fluorophore that emits in a range similar to fluorescein or rhodamine, two commonly used fluorophores for flow cytometry and fluorescence microscopy.

![Figure 2.2](image)

**Figure 2.2** – A PS-binding site in domain 1 of Anx V. Two bound PS molecules are shown coordinated to two Ca\(^{2+}\) ions. Detailed hydrogen bonding patterns are shown for those conserved residues that constitute the PS-binding consensus sequence. Adapted from Reference 99.
2.3.2 Zn\(^{2+}\)-DPA Coordination Compounds that can Detect Anionic Membranes

An appropriately designed mimic of Anx V should allow apoptotic cells to be detected with a large signal difference and simultaneously be neither toxic nor disruptive to normal biochemical processes. A small molecule mimic of Anx V should further exhibit an affinity and specificity for PS that is similar to Anx V. The utility of Zn\(^{2+}\)-2,2’-dipicolylamine (Zn\(^{2+}\)-DPA) coordination compounds as mimics of Anx V in the detection of apoptotic cells has been demonstrated.\(^\text{120}\) The Zn\(^{2+}\)-DPA complex shown below, PSS-380, was originally reported as a fluorescent sensor for phosphorylated peptides.\(^\text{121}\) The simultaneous coordination of two divalent metal cations by the organic scaffolding of PSS-380 forms a complex that is functionally similar to the Ca\(^{2+}\)-binding site of a single domain of Anx V,\(^\text{23}\) the portion responsible for membrane binding and PS recognition. The spatial separation of the two Zn\(^{2+}\) ions enables PSS-380 to interact presumably with both the carboxylate and phosphate anions present in the PS headgroup. The Ca\(^{2+}\) binding sites in Anx V differ in their affinity for Ca\(^{2+}\) in the same way that the affinity of the two Zn\(^{2+}\) binding sites in the organic scaffold of PSS-380 differ in their affinity for Zn\(^{2+}\). The first Zn\(^{2+}\) ion is bound with a K\(_a\) of approximately 10\(^7\) M\(^{-1}\), while the second Zn\(^{2+}\) is bound with an affinity of only around 10\(^4\) M\(^{-1}\).\(^\text{122}\) This
difference in cation affinity means that it is possible for PSS-380 to exist in solution predominately as the mono-Zn\(^{2+}\) form. It is then only after association of the first Zn\(^{2+}\)-DPA arm of the sensor with the anionic membrane surface that the second Zn\(^{2+}\)-DPA binding event takes place. The binding of the second Zn\(^{2+}\) eliminates PET quenching by the tertiary amine, and thus a fluorescence enhancement is observed when PSS-380 binds to an anionic membrane surface. This “three component assembly process”, consisting of the membrane surface, metal cation(s), and PS-binding group, mimics the recognition mechanism of Anx V, where only the assembly of all three components leads to a high affinity membrane-Anx V complex (Figure 2.3).

![Diagram](image)

**Figure 2.3** - The three-component assembly process that results in high affinity association of both Annexin V and Zn\(^{2+}\)-DPA coordination compounds with PS-rich membrane bilayers. Association of divalent cations, Ca\(^{2+}\) in the case of Annexin V or Zn\(^{2+}\) in the case of a Zn\(^{2+}\)-DPA coordination complex, with the anionic headgroup of a PS molecule (A), and then binding of the same metal cations by the Annexin V protein or the Zn\(^{2+}\)-DPA coordination compound (B) results in the observed high affinity interaction that facilitates PS recognition.
Apoptotic cells can be effectively identified after staining with PSS-380, and applications of PSS-380 in cell biology research have been reported for a variety of cell staining experiments, but there are limitations with its use. The anthracene fluorophore, which also serves as the sensor scaffold in this case, requires excitation at 380 nm, which falls outside the normal operational range of common flow cytometers. Further, PSS-380 suffers from rapid photobleaching, making it challenging to acquire time-dependent images.

2.3.3 Other Non-Protein PS Sensing Technologies

Functionalized quantum dots and nanoparticles have been used to monitor membrane associated biological processes, including detection of apoptosis. Schellenberger and coworkers generated a library of aminated dextran caged iron oxide nanoparticles which were subsequently functionalized with cationic peptide sequences by disulfide bond formation between the nanoparticle surface and a sidechain cysteine residue. In the best case, an 11-fold increase in fluorescence intensity was observed between normal and apoptotic cells when treated with nanoparticles functionalized with the cationic sequence RRRGRRRGC-SH. Though electrostatic interactions undoubtedly play a dominant role in the binding of these cationic nanoparticles to the apoptotic cell surface, no specific cell surface binding target was identified.

Additional strategies for detecting apoptosis based on changes in the plasma membrane have involved binding to apoptotic cells by the supramolecular assembly of cationic dye-labeled liposomes that bind anionic phospholipids. Bose and coworkers constructed cationic liposomes composed of POPC and containing the gemini surfactant...
SS-1 ((2S,3S)-2,3-dimethoxy-1,4-bis(N-hexadecyl-N,N-dimethylammonium)butane dibromide) and the fluorescent lipid analog DOPRho (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B solfonyl)). These cationic liposomes were found to homogeneously stain the cell membranes of apoptotic cells, with only slight and highly irregular binding to healthy cells. Binding of these cationic dye-labeled liposomes appears to be mediated through electrostatic interactions between the cationic gemini surfactant and the anionic PS exposed on the cell surface.

2.3.4 Other Protein-Based PS Sensing Technologies

Proteins other than Anx V have been used as sensors for apoptosis when conjugated to an appropriate fluorophore, contrast agent, or other reporter element. The C2A domain of the protein synaptotagmin I has been shown to bind anionic phospholipids in a Ca\(^{2+}\)-dependent manner, and Jung and coworkers have capitalized on this property of the C2A domain by engineering a biotinylated version of the protein which was subsequently used as an MRI contrast agent following treatment with a Gd\(^{3+}\)-chelated streptavidin conjugate. In a similar application, Heyder and coworkers designed a FITC-labeled lectin conjugate from Narcissus pseudonarcissus that could be used to detect apoptosis by exploiting the cell surface exposure of modified carbohydrate species that appear during early apoptosis. While both reporter-conjugated proteins could be used effectively in assays for apoptosis, they retain many of the same limitations of Anx V.
2.4 Summary and Directions for Research

In summary, protein-based reagents for identification of apoptotic cells, especially dye-labeled Anx V, continue to be popular. However, advances in molecular recognition and nanoparticle fabrication are rapidly making available the technology to design alternative sensing systems for detecting cell-surface PS. These improved reagents will present new opportunities for chemistry, biology, and medicine to advance our understanding of how cells and tissues function. More effective strategies for identifying apoptosis will open the door to such applications as \textit{in vivo} imaging of apoptotic tissue, designing compounds for regulation of cell cycle progression, and perhaps even development of more individualized treatment strategies for a number of common diseases. The following chapters describe the design and development of small molecule Annexin V mimics, as well as their application to PS sensing and detection of early apoptosis.
CHAPTER 3

PROPERTIES OF SECOND-GENERATION Zn\textsuperscript{2+}-DPA COORDINATION COMPLEXES

3.1 Introduction

The limitations of PSS-380 necessitated development of additional Zn\textsuperscript{2+}-DPA compounds that could be used as sensors for PS-rich membranes in detection of apoptosis and other applications where PS visualization on a cell surface could aid biomedical research or clinical medicine. The properties of a PS sensor that could find utility in a clinical environment include selectivity for PS-rich membranes over neutral membranes, an inability to cross a phospholipid bilayer, tight association with PS, good water solubility, and low cytotoxicity. These limitations and the complex chemical nature of biomembranes make the design of molecules for selective recognition of PS a challenging objective. The anionic nature of the PS headgroup makes targeting of PS-rich membranes with cationic molecules an obvious starting point for PS sensor design, but care must be taken to balance the electrostatic characteristics of a molecule with other properties, including hydrophobicity, molecular weight, and reactivity, in order to generate a workable design for a PS sensor.

The Zn\textsuperscript{2+}-DPA PS recognition unit was retained in the design of second-generation PS sensors using an “affinity group-linker-reporter element” approach.\textsuperscript{130-131} An important distinction between PSS-380 and compounds designed using this approach
is that PSS-380 is not fluorescent in the absence of an anionic membrane, while the compounds produced using the affinity group-linker-reporter element approach, where the reporter element is a fluorescent dye, would be fluorescent under all conditions. As stated earlier, the Zn$^{2+}$ binding affinity of PSS-380 is approximately $10^7$ M$^{-1}$ for the first binding event, and approximately $10^4$ M$^{-1}$ for the second binding event. This means that under normal physiological conditions, the second Zn$^{2+}$ binding site on PSS-380 would remain largely unoccupied until the mono-Zn$^{2+}$ compound, the anionic membrane, and another Zn$^{2+}$ come together simultaneously in a three-component-assembly process on the membrane surface. The binding of the second Zn$^{2+}$ then attenuates photoinduced electron transfer and the anthracene fluorophore begins to fluoresce. In the affinity group-linker-reporter element design, the Zn$^{2+}$ binding sites are far removed from the fluorescent reporter element, and photoinduced electron transfer does not quench reporter element fluorescence.

Figure 3.1 – Architectural strategy for assembling PS sensors by a modular approach.
The modularity of this design allowed each component to be individually optimized. Such a strategy is preferable to other reported techniques for recognition of anionic membranes by supramolecular complexes that would likely exhibit poor stability or increased reactivity in the complex chemical environment of the cell membrane and extracellular fluid. By employing this modular approach, a suitable PS recognition unit could be joined via an appropriate linker with a variety of fluorophores or other reporter elements to create PS sensors that could be used in a variety of applications.

The biophysical experiments described in the following sections were designed to exploit the chemical properties of the fluorescent probe NBD, which exhibits an enhancement in fluorescent emission intensity upon movement from a polar environment (the aqueous medium) to an apolar environment (the bilayer membrane interior). Furthermore, the nitro group of NBD is easily reduced to the amine by sodium dithionite, which eliminates the fluorescent properties of the molecule. Using model phospholipid bilayers designed to mimic the cell membrane, these experiments reveal fundamental biophysical properties of the second generation Zn\(^{2+}\)-DPA coordination compounds important for determining their suitability for use as PS sensors in cell-based applications.

3.2 Results and Discussion

3.2.1 Orientation of the Zn\(^{2+}\)-DPA Units on the Molecular Scaffold

The Zn\(^{2+}\)-DPA units on **PSS-380** are situated in a *para* orientation. Development of second generation Zn\(^{2+}\)-DPA based PS sensors began by determining the effect of placing the Zn\(^{2+}\)-DPA units in a *meta* orientation on a phenyl scaffold which could then
be conjugated via an appropriate linker to the reporter element NBD. Compounds 1 (provided by Dr. C. Lakshmi) and 2 (provided by Ken Stucker), where the Zn$^{2+}$-DPA units are situated, respectively, at meta and para positions, were used in phospholipid binding titration experiments to determine the binding constant for each compound to POPC vesicle membranes enriched with various amounts of POPS.

![Chemical structures of compounds 1 and 2](image)

A 1 µM solution of each compound at 25 °C was titrated with a solution of POPC vesicles (200 nm diameter) containing between 0 and 50% POPS. Binding isotherms for each compound are shown in Figure 3.2. Non-linear curve fitting of each isotherm to a 1:1 binding model$^{133}$ generated the association constants presented in Table 3.1.
Figure 3.2 – Fluorescence intensity of a 1 µM solution of 1 (top) or 2 (bottom) at 25 °C in 5 mM TES, 145 mM NaCl, pH 7.4 when titrated with POPC vesicles containing 0% (■), 5% (●), 10% (▼), 20% (▲), or 50% (♦) POPS.
TABLE 3.1

<table>
<thead>
<tr>
<th></th>
<th>POPC Only</th>
<th>50% POPS</th>
<th>20% POPS</th>
<th>10% POPS</th>
<th>5% POPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.96 ± 0.44</td>
<td>8.62 ± 1.04</td>
<td>6.59 ± 0.63</td>
<td>5.99 ± 2.29</td>
<td>3.69 ± 0.68</td>
</tr>
<tr>
<td>2</td>
<td>1.33 ± 0.73</td>
<td>8.36 ± 0.06</td>
<td>6.15 ± 0.53</td>
<td>3.70 ± 0.53</td>
<td>2.44 ± 0.87</td>
</tr>
</tbody>
</table>

NOTE: $K_{\text{association}}$ (x $10^4$ M$^{-1}$) for binding of the indicated molecule to POPC vesicles containing the indicated amount of POPS.

The fluorescence intensity of both 1 and 2 exhibited only a slight linear enhancement upon addition of a 100-fold molar excess of phospholipid when the vesicles contained only POPC. Addition of vesicles containing some fraction of POPS, however, caused an enhancement in fluorescence intensity with increasing phospholipid concentration that could be saturated at lower total phospholipid concentrations when the vesicles contained an increasing amount of POPS. Compounds 1 and 2 exhibited practically an identical binding response to vesicles enriched with all levels of POPS, indicating that the Zn$^{2+}$-DPA units can be positioned at both the meta and para positions on the phenyl scaffold without experiencing a significant difference in binding to PS-rich membranes. Based on this finding and the comparatively more convenient synthetic route to meta-oriented compounds, this orientation was chosen for the binding element with which additional second generation PS sensors were constructed.

3.2.2 Effect of Excess Zn$^{2+}$ on the Binding of Second Generation PS Sensors to Anionic Membranes

In a manner similar to **PSS-380**, the binding of Zn$^{2+}$-DPA compound 1 to anionic phospholipid membranes is expected to be a three-component-assembly process
involving a singly-bound Zn\(^{2+}\)-DPA species, an anionic membrane surface, and an additional Zn\(^{2+}\) ion. The Zn\(^{2+}\) binding affinity of 1 was expected to be similar to that reported for PSS-380, meaning that in the absence of the anionic membrane surface, significant amounts of 1 would likely exist as the singly-dissociated, mono-Zn\(^{2+}\) species. The presence of excess Zn\(^{2+}\) in the membrane environment was therefore hypothesized to lead to enhanced binding affinities due to the equilibrium shift toward the di-Zn\(^{2+}\)-DPA species that extra available Zn\(^{2+}\) should create. When 1 was titrated with 90:10 POPC-POPS vesicles in the presence of extra Zn(NO\(_3\))\(_2\), an additional enhancement in fluorescence intensity was observed relative to the case without extra Zn\(^{2+}\), but nonlinear curve fitting of the resulting binding isotherms, performed as described in 3.2.1, resulted in only a very slight enhancement in binding affinity. These binding constants are presented in Table 3.2.

### TABLE 3.2

<table>
<thead>
<tr>
<th></th>
<th>0 µM</th>
<th>1 µM</th>
<th>5 µM</th>
<th>10 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(^{2+})</td>
<td>3.70 ± 0.53</td>
<td>10.12 ± 1.35</td>
<td>8.83 ± 2.54</td>
<td>8.42 ± 0.93</td>
</tr>
</tbody>
</table>

NOTE: \(K_\text{association} \times 10^4 \text{ M}^{-1}\) for binding of 1 to vesicles consisting of 90:10 POPC-POPS in the presence of the indicated amount of excess Zn(NO\(_3\))\(_2\).

Increasing the amount of excess available Zn\(^{2+}\) from 1 µM to 10 µM had no statistically significant effect on the binding constant. This may reflect the nature of the three component assembly process, where any excess Zn\(^{2+}\) present in the system first associates with the surface of the membrane, coordinating any available anions on the membrane exterior. Such a buildup of Zn\(^{2+}\) on the membrane surface would account for
the initial jump in binding affinity that resulted from addition of only 1 µM excess Zn(NO$_3$)$_2$, and would also explain the lack of any subsequent enhancement when the free Zn$^{2+}$ levels were increased above the level apparently necessary for membrane saturation. This idea implies that the presence of higher levels of excess Zn$^{2+}$ would have a more dramatic effect on the binding of Zn$^{2+}$-DPA compounds to membranes containing an even greater fraction of anionic lipids, but such a situation is not encountered in biology, where eukaryotic cell membranes typically contain less than 20% PS.$^8$-$^9$

3.2.3 Effect of Ionic Strength on the Binding of Second Generation PS Sensors to Anionic Membranes

The extracellular fluid that baths the cell membrane is a high ionic strength environment. At the surface of a cell membrane, for example, the concentration of NaCl is approximately 150 mM. Such a high population of electrostatically charged species imparts considerable stability to other ions and charged molecules that diffuse freely throughout the extracellular fluid. Those chemical species that associate with the membrane surface purely via electrostatic interactions must therefore carry very high charge densities in order to provide a sufficient coulombic driving force for membrane binding. Membrane binding that derives purely from electrostatic interactions is sensitive to ionic strength,$^{15,134}$ and as the population of other charged species in the vicinity of the cell membrane increases, the propensity for membrane binding by the given molecule or ion is diminished.

To quantify the effect of ionic strength on binding of Zn$^{2+}$-DPA compounds to an anionic membrane surface, 1 was titrated with POPC vesicles containing 10% POPS in a
buffer containing either 100, 145, or 200 mM NaCl. The binding constants for association of 1 with the vesicle membrane are given in Table 3.3.

<table>
<thead>
<tr>
<th></th>
<th>100 mM NaCl</th>
<th>145 mM NaCl</th>
<th>200 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.33 ± 1.43</td>
<td>5.99 ± 2.29</td>
<td>2.83 ± 0.46</td>
</tr>
</tbody>
</table>

NOTE: $K_{\text{association}} \times 10^4 \text{M}^{-1}$ for binding of 1 to POPC vesicles containing 10% POPS in a buffer containing the indicated amount of NaCl.

When the buffer contained either 100 or 145 mM NaCl, essentially identical binding constants were found. Increasing the NaCl concentration to 200 mM did result in a slight decrease in the binding constant, though the value found in the case of 200 mM NaCl only narrowly satisfies the limits of statistical significance. It is worth noting, however, that increasing salt concentration should lead to decreasing binding affinity if 1 associated with a membrane surface purely by an electrostatic mechanism. The observation that 1 exhibits only a slight decrease in binding affinity when salt concentration is increased by approximately 30% (145 mM to 200 mM), coupled with the failure of 1 to bind the membrane more tightly when the salt concentration was decreased by approximately 30% (145 mM to 100 mM) suggests that the binding of 1 to the membrane surface is not governed purely by an electrostatic mechanism. Coordination of the anionic headgroup of PS by the Zn$^{2+}$ ions of 1 would require that the molecule be capable of associating with the membrane surface in a suitable orientation for PS-Zn$^{2+}$ coordination bonds to form. This requirement for a specific Zn$^{2+}$-DPA binding orientation when associating with the membrane surface provides at least a partial
explanation for the observation that the affinity of 1 for a PS-rich membrane surface appears to be independent of ionic strength.

3.2.4 Effects of Hydrophobicity of the Linker Joining the PS Recognition Unit and the Reporter Element

The Zn$^{2+}$-DPA PS recognition unit of 1 was joined to the NBD group by a four-carbon butane linker. To explore the extent to which the hydrophobicity of this linker affects the binding of the compound to PS-rich membranes, compound 3 (provided by Dr. C. Lakshmi) was prepared, where the PS recognition unit and the NBD group were separated by a more hydrophilic triethyleneoxy linker. When 3 was used in the same phospholipid titration experiments described in Section 3.2.1, the binding isotherms shown in Figure 3.3 were obtained. Fitting of these isotherms to a 1:1 binding model gave the binding constants presented in Table 3.4.

![Chemical structure of compound 3](image)
Figure 3.3 – Fluorescence intensity of a 1 µM solution of 3 at 25 °C in 5 mM TES, 145 mM NaCl, pH 7.4 when titrated with POPC vesicles containing 0% (■), 5% (●), 10% (▲), 20% (▼), or 50% (♦) POPS.

<table>
<thead>
<tr>
<th>POPC Only</th>
<th>50% POPS</th>
<th>20% POPS</th>
<th>10% POPS</th>
<th>5% POPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>5.3 ± 2.0</td>
<td>5.7 ± 0.5</td>
<td>2.2 ± 0.5</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

NOTE: $K_{association}$ ($\times 10^4$ M$^{-1}$) for binding of 3 to POPC vesicles containing the indicated amount of POPS.

The binding constants determined for association of 3 to POPC vesicles enriched with POPS bolstered the choice of the hydrophilic polyethyleneoxy linker for use in the design of later-generation PS sensors. Though the binding affinity of 3 to PS-rich membranes is slightly smaller than that of 1, the selectivity of 3 is significantly better than 1, and exhibits practically no response whatsoever to membranes containing only
POPC. The magnitude of the enhancement in fluorescence intensity observed when 3 was titrated with vesicles enriched in POPS was clearly much greater than that observed for 1. This additional selectivity for PS, apparently imparted by the more hydrophilic triethyleneoxy linker of 3, made this design the most appropriate choice for development of subsequent PS sensors.

The biophysical basis for the added PS selectivity is not obvious, though it is possible to rationalize the differences based on both the hydrophobicity and the overall length of the linker in compounds 1 and 3. In the case of 1, the very hydrophobic butyl linker provides an additional driving force for partitioning of the NBD group into the apolar interior of the bilayer. Such a driving force can facilitate insertion of the NBD group into the bilayer interior without the need for an electrostatic interaction between the Zn$^{2+}$-DPA units and an anionic membrane surface. This driving force is absent in 3, where the triethyleneoxy linker likely promotes retention of the molecule in the aqueous environment in the absence of the favorable interaction of the Zn$^{2+}$-DPA units with a PS-rich membrane surface.

Linker length must also be considered in rationalizing the differences in fluorescence enhancement and PS selectivity. The butyl linker of 1 may be only long enough to allow the NBD group to partition into the bilayer interior to the acyl region, an environment that still exhibits moderate polarity. The triethyleneoxy linker of 3, however, is twice as long as the butyl linker of 1 and may allow the NBD group to bury itself completely into the hydrocarbon region of the bilayer where the significantly lower dielectric constant may be responsible for the greater enhancement in fluorescence intensity.
3.2.5 Binding of Zn$^{2+}$-DPA Compounds to POPC Membranes Enriched in Other Anionic Lipids

The affinity of 1 and 3 for POPC membranes enriched in anionic phospholipids other than POPS was determined by titrating solutions of each molecule with POPC vesicles containing 50 % POPA or 50% POPG. The binding isotherms for titrations of a 1 µM solution of each compound in 5 mM TES, 145 mM NaCl, pH 7.4 at 25 °C are shown in Figure 3.4. Nonlinear curve fitting of these binding isotherms to the 1:1 binding model described in 3.2.1 produced the binding constants presented in Table 3.5.

<table>
<thead>
<tr>
<th></th>
<th>100% POPC</th>
<th>1:1 POPC-POPS</th>
<th>1:1 POPC-POPG</th>
<th>1:1 POPC-POPA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1</strong></td>
<td>&lt;1</td>
<td>23.3 ± 1.7</td>
<td>14.2 ± 3.8</td>
<td>11.0 ± 6.0</td>
</tr>
<tr>
<td><strong>3</strong></td>
<td>&lt;1</td>
<td>5.3 ± 2.0</td>
<td>2.0 ± 0.5</td>
<td>2.0 ± 0.3</td>
</tr>
</tbody>
</table>

NOTE: $K_{association} \times 10^{4} \text{M}^{-1}$ for binding of the indicated compound to vesicles of the indicated composition.
Figure 3.4 – Fluorescence intensity of a 1 µM solution of 1 (top) and 3 (bottom) at 25 °C in 5 mM TES, 145 mM NaCl, pH 7.4 when titrated with vesicles composed of 100% POPC (■), 1:1 POPC-POPA (▼), 1:1 POPC-POPG (▲), or 1:1 POPC-POPS (●).
For both 1 and 3, the order of binding affinities to vesicles was 1:1 POPC:POPS > 1:1 POPC:POPG ~ 1:1 POPC:POPA >> 100% POPC. The sensors were not expected to bind the zwitterionic POPC headgroup, and this titration was performed as a control to determine specificity of the sensors for anionic PS. Although 1 binds to anionic vesicles with slightly higher binding constants than 3, the more important sensing property is the difference in emission intensity for anionic vesicles versus 100% POPC. In this case, the response with sensor 3 is much more selective. For example, when the total phospholipid concentration is 60 µM, the emission of butyl-linked sensor 1 with 1:1 POPC:POPS vesicles is about two times that observed with 100% POPC vesicles, whereas the intensity ratio with the PEG-linked sensor 3 is about five (Figure 3.3).

3.2.6 Requirement of a Membrane Surface for Zn$^{2+}$-DPA Coordination Compound Binding

To be effective as sensors for PS-rich membranes, Zn$^{2+}$-DPA compounds must produce a signal when bound only to the anionic membrane surface and not to other anionic species free in solution. Since 3 exhibited the most promise of all our Zn$^{2+}$-DPA species for use as a second-generation PS sensor, this compound was tested to determine its specificity for anions found only in a membrane. Sensor 3 only responded to membrane-bound phospholipids, as evidenced by the lack of response to the short acyl chain phospholipids dihexanoylphosphatidylcholine (DHPC) and dihexanoylphosphatidylserine (DHPS), which exist as monomeric dispersions in aqueous
media. Addition of DHPC (100 µM) or a 1:1 mixture of DHPC:DHPS to a 1 µM solution of 3 results in no detectable increase in NBD fluorescence intensity (Figure 3.5).

**Figure 3.5** – Fluorescence intensity of a 1 µM solution of 3 when titrated with the short-chain phospholipids DHPC (■), and a 1:1 mixture of DHPC and DHPS (●) in 5 mM TES, 145 mM NaCl, pH 7.4. The fluorescence intensity of 3 when titrated with a 1:1 mixture of POPC-POPS vesicles (▲) is shown to illustrate the magnitude of the difference in fluorescence enhancements observed for binding of 3 to a membrane surface versus short-chain, monodisperse phospholipids.

3.2.7 Vesicle Aggregation Induced by the Zn$^{2+}$-DPA PS Affinity Group

The presence of two Zn$^{2+}$-DPA groups on the sensors evaluated thus far makes vesicle cross-linking or vesicle aggregation a possibility. To determine the extent to which the Zn$^{2+}$-DPA PS affinity group could initiate vesicle aggregation independent of any contributions from the linker or the fluorophore, POPC vesicles containing 50% POPS were titrated with the Zn$^{2+}$-DPA PS affinity group alone. Dynamic light scattering was used to determine the average particle diameter for a vesicle solution titrated with the affinity group. Vesicles exhibited an average diameter of 206 ± 65 nm in the absence of any added Zn$^{2+}$-DPA PS affinity group. The presence of increasing amounts of the
affinity group did not lead to any observed increase in particle size until the concentration of affinity group relative to the concentration of total phospholipid reached 1:1 (Figure 3.6). Importantly, the addition of Zn(NO$_3$)$_2$ alone did not lead to any increase in particle size under the same conditions in the way that would be expected for addition of Ca$^{2+}$, which is known to induce fusion of anionic membranes.$^{135}$ These findings illustrate that careful consideration of such factors as membrane cross-linking must be made when the Zn$^{2+}$-DPA PS affinity group is used as a sensor for anionic membrane surfaces, but these observations do not limit the utility of the affinity group since the relative concentrations at which aggregation was initiated can never realistically be encountered in a biological system.

3.2.8 Zn$^{2+}$-DPA Compounds Do Not Cross a Vesicle Membrane

In order to be effective as a sensor for phospholipid membranes enriched in PS, a compound must not be able to cross the membrane and gain access to the PS-rich anionic interior leaflet of healthy cells. To test whether compound 3 was capable of permeating a phospholipid membrane, the molecule was incubated with phospholipid vesicles consisting either of 100% POPC or 50% POPC-50% POPS for up to two hours. Aliquots were periodically removed from the stirring solution and treated with sodium dithionite, which chemically reduces the nitro group of the NBD fluorophore to an amine and renders the compound nonfluorescent. The NBD fluorescence emission of 3 in the presence of POPC vesicles containing either 0% or 50% POPS at various time points
Figure 3.6 – Particle size distribution of 200 nm vesicles containing 50% POPC, 50% POPS (16.7 µM total phospholipid concentration) when titrated with the Zn²⁺-DPA PS affinity group. The titration was performed in an aqueous buffer containing 145 mM NaCl, and 5 mM TES at pH 7.4.

during the incubation period is shown in Figure 3.7. In both the case of membranes containing only POPC and those containing 50% POPS, total quenching of 3 by the addition of sodium dithionite was observed, even after a two-hour incubation period. This clearly indicates that all the NBD-labeled material, the total amount of 3 present in solution, was accessible to the quenching agent. Sodium dithionite is unable to cross a
vesicle membrane, which means that none of the NBD-labeled material could have been inside the vesicle or it would not have been quenched by the addition of sodium dithionite. These results indicate that 3 is not capable of crossing either vesicle membrane over the course of two hours. This is an important observation because the possibility of performing time-based experiments requiring lengthy incubations with the Zn$^{2+}$-DPA compounds immediately becomes a valuable feature of the second-generation molecules.
Figure 3.7 – Normalized (F/F₀) fluorescence emission (530 nm) of 3 (30 µM) in a solution of POPC vesicles (25 µM total lipid) containing 0% (top) or 50% (bottom) POPS. Compound 3 was incubated for up to 120 minutes at 25 °C in an aqueous buffer containing 5 mM TES, 145 mM NaCl, pH 7.4. The superimposed emission curves in each plot correspond to NBD quenching experiments performed after incubation times of 0, 30, 60, 90, and 120 minutes. After stirring in a cuvette for 60 s, sodium dithionite was added to quench the fluorescence of all the available compound 3 that had not crossed into the vesicle interior. In each case at all time points, total quenching of fluorescence was observed.
3.3 Summary

The results of the experiments described here illustrate that Zn$^{2+}$-DPA coordination compounds can be used effectively in an “affinity group-linker-reporter element” approach to PS sensor design. By joining a phenyl scaffold bearing two Zn$^{2+}$-DPA groups oriented 1,3 to each other and a fluorescent reporter element via a hydrophilic polyethyleneglycol chain, a compound with a selective affinity for anionic membranes was created. Such a design was found to be superior to a version employing a more hydrophobic linker, as well as a version in which the Zn$^{2+}$-DPA groups were oriented 1,4 with respect to each other. Binding of the resulting molecule, compound 3, to anionic membranes was further found to occur with increasing favorability as the amount of anionic phospholipid in the membrane increased. Membrane binding was found to be basically independent of available excess Zn$^{2+}$, as the addition of Zn(NO$_3$)$_2$ did little to affect the binding constant for the tested compounds to an anionic membrane. Moreover, ionic strength was also shown to play no key role in dictating how tightly the membrane is bound by the tested compounds, signaling that membrane binding may be a function of an interaction more specific than simple coulombic attractions. These findings, as well as the observation that the molecules did not exhibit a fluorescence enhancement in the presence of non-membraneous, monodisperse lipids, speak to the likelihood of the three-component-assembly process, where a tightly bound complex is only formed in the presence of the DPA-bearing organic ligand, the anionic membrane surface, and the two Zn$^{2+}$ cations.
From a biophysical perspective, the affinity group-linker-reporter element strategy used to develop the Zn$^{2+}$-DPA compounds tested was successful in surfacing a lead candidate for PS detection, compound 3. The binding of 3 to a PS-rich membrane exhibited a binding constant on the order of $10^4$ M$^{-1}$ regardless of the PS composition, while simultaneously failing to bind a membrane containing only PC. This selectivity is ideal for use in apoptosis detection and PS recognition in a cell membrane, where the PS fraction would never likely exceed 15% of the total phospholipid. Attempts to improve the membrane binding affinity of 3, as well as its application to PS-rich cell membrane recognition are discussed in subsequent chapters.
CHAPTER 4

PROPERTIES OF A TYROSINE-Zn$^{2+}$-DPA COORDINATION COMPLEX

4.1 Introduction

The Zn$^{2+}$-DPA strategy for recognition of PS-rich phospholipid membranes could be generalized to other applications involving recognition or delivery of agents to PS-rich membranes if the PS affinity group were made more amenable to chemical conjugation techniques that would permit facile incorporation of the affinity group into larger functional scaffolds. The chemical architecture of 3 presents only a single point of attachment by which a linker or other chemical component can be appended, the phenolic oxygen. By creating a Zn$^{2+}$-DPA PS recognition molecule with an additional point of attachment, the possibility for creating more elaborate chemical species becomes realized.

The amino acid tyrosine provides an excellent starting point for development of such a building block. The phenolic oxygen of the tyrosine side chain activates the phenyl ring for installation of the Zn$^{2+}$-DPA units by electrophilic aromatic substitution. The C and N termini of the resulting unnatural amino acid are then both readily available for incorporation into more elaborate structures by traditional peptide bond-forming techniques. The report describing the initial synthesis of this compound discussed its use as a ligand in organometallic chemistry.$^{137}$ Given that the compound was already known to bind metal cations, its utility as a Zn$^{2+}$-based sensor for anionic membranes could be
easily predicted. Furthermore, the presence of the phenolic oxygen of tyrosine, which will be deprotonated to the phenoxide anion upon complexation of one or more Zn\(^{2+}\) ions, will lead to an increased affinity of the tyrosine compound for Zn\(^{2+}\) compared to the structure of molecules like 3.

Beginning with the commercially-available \(N\)-boc-tyrosine methyl ester, a compound was prepared by a Mannich-type reaction (performed by Ed O’Neil) bearing two Zn\(^{2+}\) -DPA groups.\(^{137}\) After complexation with Zn(NO\(_3\))\(_2\), compound 4 was obtained. Derivatives and higher-order variants of 4 were then used to explore the PS sensing capabilities of this design. The properties of 4 with relevance to detection of PS-rich phospholipid membranes are described here.

![Chemical structure of compound 4](image)

4.2 Results and Discussion

4.2.1 Membrane binding affinity of tyrosine-Zn\(^{2+}\) -DPA compounds

In order to perform fluorescence spectroscopy experiments, 4 was labeled with an NBD fluorophore by deprotecting the \(boc\) group of the terminal amine and forming an
amide bond to the carboxyl group of NBD-hexanoic acid. Complexation of the resulting compound with Zn(NO$_3$)$_2$ produced the fluorescent molecule 5. The binding affinity of 5 to PS-rich phospholipid membranes was determined by titrating a 1 µM solution of the complex with 200 nm POPC vesicles containing between 0 and 50% POPS. The resulting binding isotherms are shown in Figure 4.1.

![Chemical structure of 5](image)

**Figure 4.1** – Fluorescence intensity of a 1 µM solution of 5 when titrated with 200 nm diameter POPC vesicles containing 0% (■), 5% (●), 10% (▲), 20% (▼), or 50% (♦) POPS. The titrations were performed at 25 °C in an aqueous buffer containing 5 mM TES, 145 mM NaCl, pH 7.4.
Fitting the binding isotherms shown in Figure 4.1 to a 1:1 binding model gave the values for \( K_{\text{assoc.}} \) presented in Table 4.1. Compound 5 predictably bound more tightly to POPC membranes containing increasing fractions of POPS, but with binding constants slightly greater than those found for its non-phenoxy analog 1. The effect of this additional negative charge on the binding of 5 to PS-rich membranes is obviously favorable, though the binding mechanism is likely different from that hypothesized for the non-phenoxide compounds (1 and 3) described earlier.

The ionic strength experiments performed with 1 (described in Chapter 3) suggested that binding of Zn\(^{2+}\)-DPA compounds to PS-rich membranes was not entirely electrostatic, and the binding affinity of 5 for membranes enriched with PS further supports this conclusion. The overall charge on the fully-complexed version of 1 would be +4, while the overall charge on 5 would be only +3. In a binding mechanism that involved only an electrostatic attraction between the Zn\(^{2+}\)-DPA compound and the PS-rich membrane surface, compound 5, which carries 25% less cationic character, would be predicted to bind less tightly than 1. However, the three-component-assembly process by which 1 likely binds an anionic membrane must not be neglected in rationalizing the observed binding trend. Compound 1 likely exists predominantly as the singly-bound Zn\(^{2+}\) complex, meaning that until it actually binds an anionic surface and recruits a second Zn\(^{2+}\) cation to occupy its vacant DPA, 1 carries a net charge of only +2. The binding of 5 to a PS-rich membrane is almost certainly by a mechanism other than the three-component-assembly, since the phenoxide anion that results from deprotonation of the phenolic proton causes 5 to exist as the fully-complexed, di-Zn\(^{2+}\) species when free in solution. Perhaps the preorganization of the di-Zn\(^{2+}\) version of 5 is sufficient to account
for the observed enhancement in binding affinity by electrostatic means, but such a
preorganization could also be imagined to rigidify the PS-binding unit into the correct
orientation for membrane binding, therefore enhancing binding affinity by an entropic
mechanism. This question remains unanswered, though both possible factors likely
contribute to the favorability with which 5 binds a PS-rich membrane.

<table>
<thead>
<tr>
<th>POPC Only</th>
<th>50% POPS</th>
<th>20% POPS</th>
<th>10% POPS</th>
<th>5% POPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.18 ± 0.78</td>
<td>9.33 ± 1.31</td>
<td>4.12 ± 0.59</td>
<td>2.65 ± 0.36</td>
<td>1.74 ± 0.36</td>
</tr>
</tbody>
</table>

NOTE: $K_{\text{association}} \times 10^4 \text{ M}^{-1}$ for binding of 5 to POPC vesicles containing
the indicated amount of POPS.

4.2.2 Requirement of Two Zn$^{2+}$ ions in a single PS binding unit for PS selectivity

To evaluate the effect of removing one of the Zn$^{2+}$-binding portions of 5 on
association of the molecule with membranes enriched in PS, compound 6 was prepared
(Dr. Hua Jiang) and tested in vesicle titration experiments. The binding behavior of 6
was remarkably different from that of the two-Zn$^{2+}$ species 5. The fluorescence
enhancement resulting from titration of 6 with POPC vesicles containing up to 20%
POPS was basically identical to that resulting from addition of vesicles containing only
POPC (Figure 4.2). When the vesicles contained 50% POPS, however, a linear
enhancement in fluorescence intensity was observed. The net charge of 6 would be only
+1, which may explain the lack of a significant response to vesicles containing less than
50% POPS. Furthermore, the lack of a second binding arm to which a second Zn$^{2+}$ ion
could bind likely eliminates the ability of the molecule to bind selectively to a PS headgroup, moving the binding mechanism more toward an electrostatic interaction rather than a specific coordination event. Such a switch in binding modes is a possible explanation for the linear response observed for titration of 6 with vesicles containing 50% POPS. In the case of vesicles containing lesser fractions of anionic lipid, there is insufficient electrostatic interaction to initiate membrane binding by 6, and when the POPS concentration reaches the necessary threshold, 50% in this case, the binding response becomes largely electrostatic and therefore not saturable.
Clearly, a Zn\(^{2+}\)-DPA compound with one Zn\(^{2+}\) ion does not exhibit the required PS selectivity necessary for development of a workable sensor for phospholipid membranes enriched with PS, but the question of how many Zn\(^{2+}\) ions are actually necessary to observe PS selectivity, as well as their geometrical arrangement, remained unanswered. To probe this question and explore the impact of Zn\(^{2+}\) ion spatial arrangement on PS selectivity, compound 7 was prepared (synthesis by Dr. Hua Jiang), where two Zn\(^{2+}\) ions are present, just like 5, but in a fundamentally different spatial arrangement. The fluorescence behavior of 7 bears a remarkable similarity to that of 6 when titrated with POPC vesicles enriched with POPS (Figure 4.3). Again, there is no obvious selectivity for membranes enriched with anionic phospholipids, until the concentration of POPS reaches the critical threshold of 50%. At this value, the binding
response again becomes a linear function of total lipid concentration, just like the observation made with 6.

**Figure 4.3** – Fluorescence intensity of a 1 µM solution of 7 when titrated with 200 nm diameter POPC vesicles containing 0% (■), 5% (●), 10% (▲), 20% (▼), or 50% (♦) POPS. The titrations were performed at 25 °C in an aqueous buffer containing 5 mM TES, 145 mM NaCl, pH 7.4.
4.3 Summary

Construction of a Zn\(^{2+}\)-DPA coordination compound using the amino acid tyrosine as a scaffold resulted in a molecule suitable for use as a sensor for PS-rich phospholipid membranes, or incorporation into more elaborate molecules used to target anionic membrane surfaces. The extra anionic charge imparted by the deprotonated phenol of tyrosine is known to result in tighter Zn\(^{2+}\) coordination by the complex, but this property did not lead to a substantial change in membrane binding affinity when compared to the non-tyrosine versions 1 and 3. The PS selectivity of 5 makes it perfectly compatible with situations requiring detection of membrane PS under conditions relevant to actual biological applications. The binding affinity of 5 to POPC membranes containing as little as 5% POPS was found to be similar to 1 and 3. This level of PS selectivity is crucial to the success of these compounds in \textit{in vivo} applications, where the concentration of PS in a cell membrane outer leaflet is unlikely to ever exceed 15\%.\textsuperscript{136}

The absolute requirement for two Zn\(^{2+}\) ions in a single Zn\(^{2+}\)-DPA PS binding unit was illustrated by the single-Zn\(^{2+}\) species 6 and the di-Zn\(^{2+}\) species 7, both of which failed to display the same PS selectivity and binding properties as the parent molecule 5. Elimination of one Zn\(^{2+}\) ion or separation of the Zn\(^{2+}\) ions in the molecule abolished any selectivity for PS and resulted in what is likely a simple electrostatic attraction between the compounds and the anionic membrane surface in situations where the membrane contained 50% POPS.

The amino acid nature of 5 is a valuable property of this compound since both the \(N\) and \(C\) terminus can be easily deprotected and used as an attachment point for incorporation of 5 into more elaborate chemical frameworks. Standard peptide coupling
conditions could even be employed to substitute 5 into the wild type structure of proteins to probe the effects of metal coordination on protein function. The applications of 5 to PS sensing and detection of anionic membranes will be explored further in subsequent chapters, but the potential for using 5 to reveal other interesting properties of biological systems remains an area ripe for investigation.
5.1 Introduction

Tight binding is a reasonably uncommon characteristic of individual interactions between biomacromolecules. The formation of multiple weak interactions simultaneously, however, is a strategy commonly employed by nature to achieve substantial increases in the overall binding affinity between two molecules. Multivalent compounds, those molecules capable of simultaneously forming multiple interactions with a binding partner, commonly exhibit very tight binding constants even though the individual contributions to the overall binding affinity may each be small.

Multivalency and cooperativity are important themes in the supramolecular chemistry of biological systems, including interactions of natural and designed compounds with a phospholipid membrane. Cell-surface carbohydrates, for example, are often arranged in discrete patterns on the membrane, making it possible for a multivalent binding partner to interact with several carbohydrates at once. This effect, termed the “cluster glycoside effect,” results in substantial enhancements in binding affinity for many of the galectins, or carbohydrate-binding proteins. The effect of a membrane bilayer on ligand binding by synthetic multivalent molecules embedded in the membrane has also been described. The observed association constants for ligand
binding by membrane-embedded multivalent receptors were significantly enhanced relative to the observed solution-phase values, and additional receptor-to-ligand binding ratios were discovered that did not occur in solution.

The effect of a membrane surface on the ligand binding ability of a multivalent compound is still a developing area of research. Until now, most reports describing the interaction of a multivalent compound with a ligand on the membrane surface have focused on proteins or carbohydrates,\textsuperscript{144-145} ligands that only decorate the surface of the phospholipid bilayer. While multivalent compounds that interact with the phospholipids themselves have not been described to any extent in the literature, the high local concentration of phospholipids in the environment of the membrane represents an excellent opportunity to apply the principles of multivalency to cell surface recognition.

In the classical sense, enhancements in binding affinity that arise from multivalent interactions between a molecule and a ligand result from the added enthalpic gains associated with multivalency.\textsuperscript{146} Assuming that the total number of interactions between a multivalent compound and its ligand(s) occur sequentially, the substantial entropic penalty associated with bring two molecules together is essentially covered by formation of the first interaction. Formation of all subsequent interactions simply results in added enthalpic stabilization of the initially-formed complex since no substantial entropic burden remains to be overcome after the initial interaction is formed. From the kinetic perspective, multivalency leads to enhancements in binding affinity by decreasing the rate of $k_{off}$, or the rate of dissociation for the compound-ligand complex. Assuming that the rate of complex formation between two compounds, $k_{on}$, is similar for all receptor-ligand interactions of a given type, regardless of valency, the formation of additional
interactions between the two molecules following their initial interaction can then be thought to stabilize the resulting compound-ligand complex simply by providing an additional number of interactions that must simultaneously be disrupted in order for the complex as a whole to dissociate.

Based on what was learned about the binding of Zn$^{2+}$-DPA compounds that coordinate two Zn$^{2+}$ cations to the cell membrane, a logical step toward developing sensors for PS-rich membranes was the synthesis of multivalent derivatives that could simultaneously coordinate more Zn$^{2+}$ ions and likely exhibit a binding affinity for PS-rich membranes that would be greater than the monomeric compounds previously described. Two different classes of multivalent Zn$^{2+}$-DPA compounds have been prepared, one based on a non-peptide, glutamic acid framework, and the other, a polypeptide, based on tyrosine. The properties of these compounds and their ability to detect PS-rich phospholipid membranes are described in this chapter.

5.2 Multivalent Second-Generation Zn$^{2+}$-DPA PS Binding Compounds

Three additional NBD-labeled-Zn$^{2+}$-DPA coordination compounds were tested to determine if the presence of additional Zn$^{2+}$ ions in a molecule would lead to increased PS selectivity or enhancements in PS binding affinity. Compound 1, which binds two Zn$^{2+}$ ions in the fully bound form, was compared with compounds 8, 9, and 10, which coordinate one, four, and eight Zn$^{2+}$ ions, respectively, in their fully bound forms. These multivalent Zn$^{2+}$-DPA compounds were prepared (Dr. C. Lakshmi) using a glutamic acid scaffold, which allowed the compounds to be conveniently conjugated to the NBD fluorophore. The hydrophobicity of the amino acid scaffold from which the compounds
were constructed makes them more appropriate for comparison with 1 than with the more hydrophilic 3. Each compound was titrated with POPC vesicles containing between 0 and 50% POPS in order to produce binding isotherms that were then used to determine association constants for each compound to the PS-enriched vesicle membranes. Binding isotherms for each compound are shown in Figure 5.1.
Figure 5.1 – Fluorescence intensity of a 1 µM solution of 8 (top), 9, (middle), and 10 (bottom) at 25 °C in 5 mM TES, 145 mM NaCl, pH 7.4 when titrated with POPC vesicles containing 0% (■), 5% (●), 10% (▼), 20% (▲), or 50% (♦) POPS.
A noticeable feature of these binding isotherms, and the isotherm already shown for 1, is the similarity with which each molecule responds to PS-rich vesicle membranes. Regardless of the number of potential Zn$^{2+}$ binding sites in the molecule, one for 8, two for 1, four for 9, and eight for 10, the amount of phospholipid required to reach saturation of fluorescence emission intensity remains basically constant. That is, when vesicles contained 20% POPS, the amount of total phospholipid required to saturate the binding of 1 was practically the same amount required to saturate 10, even though the number of potential PS binding sites is four-fold greater. The binding constants derived from these isotherms are presented in Table 5.1.

### TABLE 5.1

<table>
<thead>
<tr>
<th>Zn$^{2+}$ Binding Sites</th>
<th>POPC Only</th>
<th>50%</th>
<th>20%</th>
<th>10%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1</td>
<td>3.19 ± 0.55</td>
<td>18.00 ± 0.001</td>
<td>12.50 ± 5.80</td>
<td>12.60 ± 5.90</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>0.96 ± 0.44</td>
<td>8.62 ± 1.04</td>
<td>6.59 ± 0.63</td>
<td>5.99 ± 2.29</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>0.13 ± 0.10</td>
<td>9.81 ± 0.79</td>
<td>7.47 ± 1.26</td>
<td>4.41 ± 1.83</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>0.58 ± 0.32</td>
<td>9.14 ± 1.96</td>
<td>6.82 ± 2.97</td>
<td>6.26 ± 2.91</td>
</tr>
</tbody>
</table>

NOTE: $K_{association}$ (x $10^4$ M$^-1$) for binding of the indicated molecule to POPC vesicles containing the indicated amount of POPS.

The substantial binding constant determined for association of 8 with membranes containing only POPC further supported the initial hypothesis that a Zn$^{2+}$-DPA compound must coordinate at least two Zn$^{2+}$ ions in order to exhibit any genuine selectivity for PS-rich membranes. However, even more noteworthy is the binding constant consistency exhibited by all the molecules for a given membrane composition. If compound 8 is not considered, the remaining Zn$^{2+}$-DPA compounds exhibit a
practically identical value for $K_{assoc.}$ for all PS fractions. This is a striking example of the failure of multivalency to lead to enhancements in membrane binding affinity by a molecule designed to simultaneously present multiple ligand binding groups. Similar behavior is exhibited by the tyrosine-Zn$^{2+}$-DPA complexes, discussed in more detail below.

5.3 Multivalent Tyrosine-Zn$^{2+}$-DPA PS Binding Compounds

The peptide character of the tyrosine-Zn$^{2+}$-DPA compounds discussed in Chapter 4 immediately suggested the possibility of assembling higher-order multivalent compounds through the formation of peptide bonds linking tyrosine-Zn$^{2+}$-DPA monomers in a linear fashion. Compounds with one, two, four, and eight Zn$^{2+}$ ions were prepared on the tyrosine-Zn$^{2+}$-DPA scaffold, which are compounds 6, 5, 11, and 12, respectively.
Each of these molecules was titrated with POPC vesicles enriched with 0 to 50% POPS in the same manner as described earlier. The binding isotherms for 11 and 12 resulting from these titrations are shown in Figure 5.2. These data also raise interesting questions about multivalency and phospholipid selectivity. The necessity of two Zn$^{2+}$ ions in the same tyrosine monomer for PS selectivity was discussed in Chapter 4, but here we see evidence that simply having the capacity to bind additional Zn$^{2+}$ ions is not sufficient to guarantee PS selectivity. The fluorescence enhancement exhibited by 11 when titrated with vesicles containing only POPC, for example, is similar to the response generated when the vesicles contained 5% POPS. The four Zn$^{2+}$ ions of 11 are therefore
not capable of guaranteeing added PS selectivity. Binding constants determined from these isotherms are presented in Table 5.2.

![Fluorescence Intensity vs. Total Phospholipid Concentration](image)

**Figure 5.2** – Fluorescence intensity of a 1 µM solution of 11 (top) and 12 (bottom) at 25 °C in 5 mM TES, 145 mM NaCl, pH 7.4 when titrated with POPC vesicles containing 0% (■), 5% (●), 10% (▼), 20% (▲), or 50% (♦) POPS.
TABLE 5.2

<table>
<thead>
<tr>
<th>Zn$^{2+}$ Binding Sites</th>
<th>POPC Only</th>
<th>50%</th>
<th>20%</th>
<th>10%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1</td>
<td>2.18 ± 0.78</td>
<td>9.33 ± 1.31</td>
<td>4.12 ± 0.59</td>
<td>2.65 ± 0.36</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2.18 ± 0.78</td>
<td>9.33 ± 1.31</td>
<td>4.12 ± 0.59</td>
<td>2.65 ± 0.36</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>3.72 ± 1.27</td>
<td>15.60 ± 1.20</td>
<td>8.70 ± 0.65</td>
<td>6.46 ± 1.44</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>1.57 ± 0.35</td>
<td>13.38 ± 1.13</td>
<td>3.94 ± 1.04</td>
<td>1.26 ± 0.22</td>
</tr>
</tbody>
</table>

NOTE: $K_{association}$ ($x 10^4 M^{-1}$) for binding of the indicated molecule to POPC vesicles containing the indicated amount of POPS.

In the case of 11 and 12, slightly tighter binding to PS-rich vesicles was observed relative to the monomeric compound 5, but with a loss of PS selectivity by 11. Though there was an enhancement in binding affinity, the gains were slight and fell far short of the substantial enhancements previously reported for other designed multivalent systems.

5.4 Rationalizing the Lack of a Multivalency Effect

Multivalency-derived enhancements in binding affinity are traditionally ascribed to the added favorable enthalpy of subsequent ligand-molecule interactions that form after the initial binding event that joins the multivalent molecule and its first ligand. These interactions can form with a greatly decreased entropic penalty relative to the first interaction, and the favorability of these ligand-binding events therefore manifests itself in the substantial enhancements in ligand binding affinity often observed for multivalent systems. In the Zn$^{2+}$-DPA systems considered here, however, multivalency did not lead to the expected enhancements in membrane binding affinity. A number of possible explanations can be offered to rationalize these observations, including incomplete Zn$^{2+}$
complexation by the multivalent molecules, conformational restrictions in the molecules resulting from membrane binding, and various forms of enthalpy-entropy compensation.

The initial expectations of a multivalency effect were based entirely on the notion that all the available DPA binding sites would be occupied by $\text{Zn}^{2+}$ ions. Results from later studies and reports from other groups have shown this assumption to be largely false for compounds 1, 9, and 10, and the extent to which it is true for the tyrosine compounds 11 and 12 remains uncertain. Regardless, any unoccupied DPA $\text{Zn}^{2+}$ binding site would detract from the expected multivalency effect, and such a possibility is a genuine concern in the present investigation.

Increases in effective concentration of the ligand in the environment of the binding units of a multivalent receptor are often invoked as an explanation for any observed multivalency effect leading to increased binding affinity. Such explanations are entirely reasonable in the context of static ligands, such as association of a multivalent small molecule with ligands immobilized on a surface. The effective concentration argument has been used well to explain binding affinity enhancements of up to three orders of magnitude in exactly these situations.\textsuperscript{147-149} The case of PS-rich membrane binding by cationic proteins or small molecules, however, is fundamentally different from the previously described model systems in two important ways. First, the composition of the membrane is not homogeneous; there are components other than PS present. Second, and perhaps more importantly, the dynamics of the membrane are such that the PS headgroups do not occupy a spatially addressable locality in the membrane, but rather diffuse freely throughout the bilayer leaflet in which they are found. A consequence of these properties is that PS binding by a multivalent species, whether
protein or small molecule, results in both a sequestering of PS into discrete domains in a single membrane leaflet, as well as a restriction in the two-dimensional lateral mobility of each individual PS molecule. Association of Annexin V with a PS-rich membrane is known to induce lateral ordering of PS molecules, and the multivalent \( \text{Zn}^{2+} \)-DPA compounds tested here could logically be expected to cause similar effects.

The ordering of PS molecules in a membrane leaflet is an entropically disfavorable process, and any gain in favorable enthalpy of binding associated with multivalency may be negated by this disfavorable contribution from binding entropy. Examples of such entropy-enthalpy compensation have been suggested by others, though some researchers continue to question the actuality of the entropy-enthalpy compensation phenomenon. Beyond any ordering of the phospholipids that may take place upon membrane binding, the rigidification of the multivalent receptors themselves, particularly \( 9 \) and \( 10 \), that would likely occur upon membrane binding presents another area where disfavorable entropic effects could result in a failure of multivalency to produce the expected enhancements in binding affinity.

5.5 Summary

\( \text{Zn}^{2+} \)-DPA coordination compounds with the capacity to bind increasing numbers of \( \text{Zn}^{2+} \) ions failed to display the enhancements in membrane binding affinity often associated with multivalency. While no clear explanation for this failure could be gleaned from the available data, the fact that both the tyrosine-based and the glutamic acid-based multivalent compounds behaved similarly suggests that the lack of multivalency in these cases is likely due to a general property of membrane binding
rather than a particular characteristic of any molecule studied here. The absence of any tangible benefit of multivalency in PS recognition by Zn$^{2+}$-DPA coordination compounds indicated that the original design bearing two Zn$^{2+}$DPA units was suitable for continued use in developing sensors to be used in cellular applications.
CHAPTER 6

INDICATOR DISPLACEMENT ASSAYS FOR DETECTION OF PS-RICH PHOSPHOLIPID MEMBRANES

6.1 Introduction

The commonly used receptor-spacer-reporter element approach to sensor design has resulted in development of many valuable molecules for detecting important analytes. However, despite the utility of this approach, the resulting sensors are inherently limited by the synthetic effort necessary to link the receptors to the various reporter elements that often are needed when the sensing compounds are used in real-world sensing applications. A possible solution to the problem presented by these necessary synthetic demands is the indicator displacement strategy, where a receptor is bound noncovalently to a fluorogenic or chromogenic indicator whose spectral properties change upon complexation with the receptor (decrease in fluorescence intensity, shift in emission or absorption maximum, etc.). In the presence of the analyte, the receptor-indicator complex dissociates and the receptor binds to the analyte, restoring the spectral properties of the indicator. The most attractive feature of the indicator displacement approach is the lack of synthetic effort required to assemble the receptor-indicator complex. A single receptor for a given analyte can be used to prepare numerous
receptor-indicator complexes, where the only requirement is that the receptor form a noncovalent complex with the chosen indicator(s).

A number of indicator displacement assays have been developed for detection of a wide variety of chemical species. In the most common applications of the indicator displacement technique, the receptor molecule, frequently a metal complex which coordinates two metal cations, binds a neutral or anionic dye, which is then displaced in the presence of the analyte. Often the analyte is a polyatomic anion, such as carboxylate or phosphate, and the assays are normally discussed in terms of their ability to detect the analyte in solution. The headgroup of PS is formally a polyatomic anion, and indicator displacement assays for phosphate and carboxylate, both of which are found in the PS headgroup, have been described. With this in mind, a variety of indicator displacement assays were developed to detect PS in a phospholipid membrane environment.

The PS receptors and indicators shown in Figure 6.1 were combined to produce chemosensing ensembles that were then titrated with aqueous solutions of anions, as well as POPC vesicles enriched with POPS. This chapter describes the performance of these noncovalent chemosensing ensembles in assays for phosphate oxyanions and PS-rich phospholipid membranes.

6.2 Results and Discussion

6.2.1 Receptor-Indicator Binding Constants

To determine an association constant for receptor-analyte binding using the indicator displacement method, a value for the receptor-indicator binding constant is necessary. Beginning with the UV dye pyrocatechol violet, 17, a series of UV titration
experiments were performed in which an aqueous solution of the dye was titrated with a solution of each receptor 13-16. Since the absorbance of 17 was strongly dependent on

Figure 6.1 – Structure of the PS receptors (13 – 16) and the indicators (17– 19) used in the indicator displacement assays for phosphate oxyanions and PS-rich membranes.
concentration, each titrant receptor solution was prepared containing 50 µM 17, which was equal to the dye concentration in the solution into which the receptor was added. The addition of each receptor resulted in a decrease in absorbance of 17 at wavelengths between 380 and 500 nm and an increase in absorbance at wavelengths between 500 and 750 nm, with an isosbestic point at approximately 500 nm. The UV absorbance spectra of 17 resulting from these titrations are shown in Figure 6.2.

Figure 6.2 - UV-Visible absorbance spectra of 17 (50 µM) when titrated with Zn$^{2+}$-DPA complexes 13 (A), 14 (B), 16 (C), or 15 (D) over the concentration range 0 to 400 µM. Titrations were performed at 25 °C in 5 mM TES buffer, 145 mM NaCl, pH 7.4. The spectra in each panel correspond to receptor concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, 300, 350, and 400 µM, respectively.
The receptors were also titrated into an aqueous solution of the fluorescent coumarin compound 18 in order to determine association constants for these interactions. The methylsulfonate substituent on 18 was necessary in order to confer water solubility, which made this compound suitable for experiments in aqueous solution. Poor water solubility eliminated the parent coumarin compound 19 from consideration as a potential indicator because the primary purpose of the experiments was to develop a sensing ensemble that could be applied in a physiologically relevant setting. Binding of each receptor to 18 resulted in a quenching of indicator fluorescence. Quenching of 18 by the various receptors is illustrated by the curves shown in Figure 6.3.

Figure 6.3 - Fluorescence intensity at 480 nm (I/I₀) of a 10 μM aqueous solution of 18 in 5 mM TES, 145 mM NaCl, pH 7.4. Fluorescence intensity was quenched by addition of 13 (■), 14 (●), 16 (▲), and 15 (▼) over the concentration range of 0 to 100 μM.
The UV absorption spectra shown in Figure 6.2 and the fluorescence quenching curves shown in Figure 6.3 were fit to a 1:1 binding model in order to determine the association constants presented in Table 6.1.

### Table 6.1

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Indicator 17</th>
<th>Indicator 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>&gt;17</td>
<td>231 ± 160</td>
</tr>
<tr>
<td>14</td>
<td>&gt;17</td>
<td>60.2 ± 15.4</td>
</tr>
<tr>
<td>15</td>
<td>17.6 ± 5.4</td>
<td>10.3 ± 0.6</td>
</tr>
<tr>
<td>16</td>
<td>5.6 ± 2.2</td>
<td>17.8 ± 3.0</td>
</tr>
</tbody>
</table>

NOTE: $K_{\text{association}} \times 10^4 \text{M}^{-1}$ for receptor-indicator binding. Each value represents the average of at least three separate experiments. All binding constants were determined at 25°C in aqueous solutions of 5 mM TES, 145 mM NaCl, pH 7.4.

The dinuclear Zn$^{2+}$-DPA compounds 13 and 14 bound most tightly to both indicators tested, which is not surprising given that the phenoxide anion of 16 provides one coordination partner for each of its Zn$^{2+}$ ions, and the single-Zn$^{2+}$ compound 15 can provide only a single metal cation to interact with the catechol functionalities of both indicators, which is presumably where each receptor binds the indicators, though association with the sulfonate anion of 18 cannot be ruled out. The 1,3-oriented Zn$^{2+}$-DPA compound 13 bound the fluorescent coumarin sulfonate 18 much more tightly than any of the other receptors, and the effect of this interaction is clearly visible in both the
UV absorption and the fluorescence emission spectra of 18 when titrated with an aqueous solution of 13. The UV absorbance of 18 exhibits a clear isosbestic point at approximately 390 nm (Figure 6.4). Additionally, the fluorescence emission maximum of 18 at 480 nm is markedly decreased in intensity with addition of increasing amounts of 13 (Figure 6.5). The validity of the 1:1 binding model assumed for binding of 18 by the Zn$^{2+}$-DPA species tested was proven by performing a Job’s plot analysis (continuous variations) for binding of 18 by 13. The quenching curve and the associated Job’s plot shown in Figure 6.6, where 13 was used as the receptor, illustrate clearly that the binding stoichiometry is indeed 1:1.

**Figure 6.4** - UV-Vis spectra of 18 following the addition of increasing amounts of 13. Compound 13 was titrated into a 100 µM solution of 18 over the concentration range 0 to 250 µM. All titrations were performed at 25 °C in aqueous solution, pH 7.4, 5 mM TES, 145 mM NaCl. Spectra correspond to receptor concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, and 250 µM, respectively.
Figure 6.5 - Fluorescence emission spectra of 18 following the addition of increasing amounts of 13. Compound 13 was titrated into a 10 µM solution of 18 over the concentration range 0 to 100 µM. Excitation wavelength was 347 nm. All titrations were performed at 25 °C in aqueous solution, pH 7.4, 5 mM TES, 145 mM NaCl. Spectra correspond to receptor concentrations of 0, 1, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, and 100 µM, respectively.

Figure 6.6 - Fluorescence intensity of 18 at 480 nm in the presence of increasing amounts of 13. Excitation wavelength was 347 nm. INSERT: Jobs plot for the receptor-indicator binding of 18 and 13, clearly indicating a 1:1 stoichiometry. All experiments were performed at 25 °C in aqueous solution, pH 7.4, 5 mM TES, 145 mM NaCl.
6.2.2 Phosphate Oxyanion Detection by Indicator Displacement

The principle objective of this research was to develop an indicator displacement assay for the detection of PS-rich phospholipid membranes. In order to expedite the process, each receptor was first complexed with 18 and titrated with pyrophosphate or hydrogenphosphate in order to determine to a first approximation whether the given receptor-indicator complex was suitable for further testing with phospholipid vesicles. The choice of 18 as the indicator came largely from the desirability of a fluorescence-based assay in order to access the lower concentrations of complex necessary and the lower limits of analyte detection associated with fluorescence assays in comparison with their UV counterparts.

Each receptor-indicator complex (10 µM total concentration of each component) was titrated with an aqueous solution of either sodium pyrophosphate or sodium hydrogenphosphate. These anions were chosen to simulate the anionic component of a PS headgroup that the sensors would encounter in a PS-rich membrane. The ensembles formed by compounds 13 and 16 were found to be the only receptor-indicator combinations capable of recognizing pyrophosphate or hydrogenphosphate. Treatment of each receptor-indicator complex with either pyrophosphate or hydrogenphosphate resulted in a concentration-dependent restoration of fluorescence by 18. A representative plot of the fluorescence emission spectra of 18 when pyrophosphate was added to the 13/18 complex is shown in Figure 6.7. The fluorescence emission intensity at 480 nm acquired in each titration could be used in the standard mathematical treatment for displacement assays\textsuperscript{172} to determine the association constants presented in Table 6.2 for binding of receptors 13 and 16 to both pyrophosphate and hydrogenphosphate. The
association constant determined for 16 with hydrogenphosphate, \((11.4 \pm 3.3) \times 10^4 \text{ M}^{-1}\), agrees remarkably well with the previously reported\textsuperscript{166} value of \((11.2 \pm 0.8) \times 10^4 \text{ M}^{-1}\). The association constant for 16 with pyrophosphate, \((6.7 \pm 1.8) \times 10^5 \text{ M}^{-1}\), also agrees well with previous reports\textsuperscript{160} after consideration of the greater ionic strength used in the present studies.

Figure 6.7 - Fluorescence emission spectra of 18 after addition of increasing amounts of sodium pyrophosphate to a 1:1 mixture of 18 and 13 (concentration of the receptor-indicator complex was 10 µM). Excitation wavelength was 347 nm. All experiments were performed at 25°C in aqueous solution, pH 7.4, 5 mM TES, 145 mM NaCl. Spectra correspond to pyrophosphate concentrations of 0, 1, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, and 100 µM, respectively.
TABLE 6.2

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Pyrophosphate</th>
<th>Hydrogenphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>156 ± 26</td>
<td>7.3 ± 3.7</td>
</tr>
<tr>
<td>16</td>
<td>67.2 ± 18.2</td>
<td>1.1 ± 0.3</td>
</tr>
</tbody>
</table>

NOTE: $K_{\text{association}}$ for receptors and the indicated phosphate oxyanions ($x \times 10^5 \text{ M}^{-1}$). All titrations were performed at 25 °C in aqueous solutions buffered at pH 7.4 with 5 mM TES, 145 mM NaCl. Each value represents the average of at least three separate experiments.

Paralleling the association constants determined for each receptor-indicator binding interaction, 13 bound each anion more strongly than 16. This may again reflect the greater cationic character of 13, which would be +4 in its fully-bound form, while 16 would only be +3. The binding of each receptor was considerably stronger to pyrophosphate than to hydrogenphosphate, by more than 100 fold in each. This likely reflects the greater anionic character of pyrophosphate, and coulombic attractions are a plausible rationalization for this observed binding strength.

6.2.3 Detection of PS-rich Phospholipid Membranes by Receptor-Indicator Complexes

In order to truly assess the utility of the indicator displacement assay for detection of PS-rich phospholipid membranes under conditions applicable to cellular systems,
vesicles mimicking the cell membrane were prepared and titrated into various receptor-indicator complexes. In order to be useful as a sensor for early apoptosis, the chemosensing ensemble would be required to detect low levels of PS in a membrane (Figure 6.8) and produce no response when treated with membranes containing only zwitterionic phospholipids. Receptors 13-16 were each mixed in 1:1 ratios with either of the indicators 17 and 18 with the hope of developing a UV-visible and fluorescence assay, respectively, for membrane PS. In the most successful example, POPC membranes containing as little as 5% POPS could be detected using the indicator displacement technique.

The receptor-indicator complexes produced using the UV-visible dye 17 presented what may be the most successful of the receptor-indicator displacement ensembles for detecting membrane PS. The 16/17 complex was actually capable of

![Diagram](image)

**Figure 6.8 - Displacement assay for apoptosis:** Following phosphatidylserine externalization to the outer membrane monolayer, the indicator (I) is displaced from the receptor-indicator complex (R-I) by the phosphatidylserine headgroup, generating a detectable signal by restoration of the spectral properties of the indicator.
visually reporting the presence of PS in a phospholipid membrane (Figure 6.9). Upon addition of the Zn\(^{2+}\)-DPA complexes to an aqueous solution of 17, the color of the solution changed immediately from an orange to a deep blue. The sensing ensemble that employed receptors 13 - 16 and indicator 17 were found to easily detect membrane PS when the fraction of PS in the membrane was 50%. The ensembles formed between 17 and the Zn\(^{2+}\)-DPA receptors 13, 14, and 15 were not able to detect POPC membranes containing any lesser fraction of PS, but the 16/17 ensemble, however, was able to distinguish between membranes containing only POPC and those containing lesser fractions of POPS. When treated with POPC vesicles containing 20% POPS, the 16/17 ensemble underwent a color change visible to the naked eye, and when the vesicles contained as little as 5% POPS, the color change could still be detected spectroscopically (Figure 6.10).

![Figure 6.9](image)

**Figure 6.9** - Aqueous solutions of the 16/17 ensemble (50 µM each) in the presence of POPC vesicles containing various amounts of POPS. (A) Indicator 17 only. (B) –(F) are solutions of the 16/17 ensemble treated with POPC vesicles containing 0, 5, 10, 20, and 50% POPS, respectively. (G) The 16/17 ensemble in the absence of any phospholipids. All solutions contain 5 mM TES, 145 mM NaCl, pH 7.4.
Figure 6.10 - UV-Visible absorbance spectra for titration of a 50 µM 1:1 complex of 16 and 17 with phospholipid vesicles containing 95% POPC – 5% POPS. Only the 16/17 ensemble was able to detect vesicles containing 5% POPS. Titrations were performed at 25 °C in 5 mM TES buffer, 145 mM NaCl, pH 7.4. Spectra correspond to total phospholipid concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, 300, 350, and 400 µM, respectively.

We hypothesized that the anionic headgroup of phosphatidylserine could also be detected in a fluorescent displacement assay, giving us the advantage of the lower concentrations associated with fluorescent indicators. Titration of Zn$^{2+}$-DPA compounds 13-16 into an aqueous solution of 18 resulted in a concentration-dependent quenching of the indicator fluorescence, as described earlier. In the case of 13, the fluorescence intensity of 18 was quenched by approximately 75% after addition of a single equivalent of 13, and by approximately 90% when a second equivalent was added. Compounds 14-16 each exhibited similar, though less dramatic effects on the fluorescence of 18.

Equimolar amounts of receptor and indicator 18 were mixed in a cuvette (10 µM each) and stirred for approximately one minute. POPC vesicles containing from 0 to 50% POPS were then titrated into the solution and the fluorescence intensity was
observed ($\lambda_{\text{ex.}} = 347 \text{ nm}, \lambda_{\text{emiss.}} = 480 \text{ nm}$). The indicator-Zn$^{2+}$-DPA complexes of 13, 14, and 16 responded to addition of vesicles containing POPS with an increase in fluorescence intensity, while the complex of 15 failed to respond with detectable enhancements. In each case, vesicles containing 50% phosphatidylserine elicited the most dramatic enhancement in fluorescence intensity (Figure 6.11). The increase in fluorescence intensity upon addition of vesicles containing 50% POPS was sufficient to generate saturated binding curves in the case of 13 and 16, while 14 exhibited a linear enhancement with increasing phospholipid concentration. After addition of 2.5 equivalents of phospholipid to the 13/18 ensemble, a 1.8 fold enhancement in fluorescence intensity was observed. Similarly, 2.5 equivalents of phospholipid resulted in 1.2 and 1.1 fold enhancements for the ensembles of 14/18 and 16/18, respectively.

Vesicles containing lesser amounts of POPS were detectable by the 13/18 ensemble, which exhibited enhancements in fluorescence intensity when treated with vesicles containing as little as 5% POPS. The 14/18 ensemble could detect vesicles containing as little as 20% POPS, while the 16/18 ensemble exhibited no significant response to vesicles containing less than 50% POPS. Phospholipid-Zn$^{2+}$-DPA complex binding constants derived from these titrations are summarized in Table 6.3. Importantly, these fluorescent ensembles also failed to respond to treatment with vesicles enriched in the anionic phospholipids POPA and POGP, and again required a membrane surface for POPS recognition as evidenced by the lack of response to treatment with a DHPS suspension.
Figure 6.11 - Fluorescence intensity of 18 as a function of total phospholipid concentration when vesicles of the indicated composition were titrated into a 10 µM solution of a 1:1 complex with 13 (A), 14 (B), or 16 (C). POPC liposomes contained 0% (♦), 50% (●), 20% (▲), 10% (▼), or 5% (■) POPS. Titrations were performed at 25 °C in 5 mM TES buffer, 145 mM NaCl, pH 7.4.
### TABLE 6.3

<table>
<thead>
<tr>
<th>Receptor</th>
<th>POPC Only</th>
<th>50% POPS</th>
<th>20% POPS</th>
<th>10% POPS</th>
<th>5% POPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>-</td>
<td>55.5 ± 14.0</td>
<td>94.6 ± 31.8</td>
<td>10.2 ± 9.6</td>
<td>3.0 ± 1.8</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>1.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>0.6 ± 0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

NOTE: $K_{\text{association}} \times 10^5 \text{ M}^{-1}$ for receptor binding to POPC membranes containing the indicated amount of POPS. Each value represents the average of at least three separate experiments. All binding constants were determined at 25 °C in aqueous solutions of 5 mM TES, 145 mM NaCl, pH 7.4.

### 6.3 Summary

Indicator displacement assays have been developed that allow vesicle membranes enriched in the anionic phospholipid POPS to be detected under physiological conditions, in certain cases with the naked eye. Importantly, none of the sensing ensembles described respond to monodisperse phosphatidylserine, underscoring the necessary requirement of a membrane surface for successful indicator displacement, and hence phosphatidylserine recognition. The assays can detect phosphatidylcholine vesicles containing as little as 5% phosphatidylserine. Therefore, these displacement assays may be useful for detecting the onset of cell apoptosis, where the fraction of phosphatidylserine exposed on the cell surface increases to around 10% (clustering may lead to patches of membrane with significantly higher localized concentrations).[^136][^173]

Further, the simple non-covalent assembly of these ensembles makes high throughput screening a clear possibility.
CHAPTER 7
DETECTION OF EARLY APOPTOSIS USING SECOND GENERATION ZN\(^{2+}\)-DPA
COORDINATION COMPOUNDS

7.1 Introduction

In healthy eukaryotic cells, phosphatidylserine (PS) usually constitutes less than 10% of the total phospholipid in the membrane and is confined almost exclusively to the inner monolayer.\(^9\) During the early stages of programmed cell death (apoptosis), the phospholipid distribution is scrambled, which leads to the appearance of PS on the cell surface. A range of annexin V-dye conjugates are now commercially available and used extensively in cell biology research for detecting externalized PS, which signals the onset of apoptosis. However, annexin V has some drawbacks that diminish its utility in certain applications. For example, the protein may not have the necessary chemical stability for employment in high-throughput screening of cancer drugs,\(^{174}\) and may lack the biochemical stability necessary for \textit{in vivo} imaging of dying tissue.\(^{115-116}\) Other potential problems are the slow kinetics of membrane binding,\(^{114}\) and the calcium dependence, which can potentially lead to artifacts such as activation of non-specific membrane scramblase activity.\(^60\) It is apparent that a small molecule substitute for annexin V that binds PS-rich membranes in the same manner as annexin V but without the limiting characteristics of the protein would be a valuable contribution to cell biology research.
Initial reports that fluorescent Zn$^{2+}$-dipicolylamine (DPA) coordination complexes with a selective affinity for membrane surfaces enriched in anionic PS revealed that they can be used to detect apoptosis. These initial studies used fluorophores with non-optimal properties, but the work produced important structural information which led to an understanding of the modular, three component assembly process. A similar design has been employed in the preparation of the compounds described here, which contains a PS affinity group (two Zn$^{2+}$-DPA subunits that are oriented meta on a phenyl ring) that is attached via a tris(ethyleneoxy) linker to a reporter element. This design has produced some examples of workable PS sensors incorporating practical reporter elements suitable for use in actual biological research. Specifically, the utility of the fluorescein derivative PSS-480 (Phosphatidylserine Sensor – 480 nm excitation) in fluorescence microscopy and flow cytometry experiments is discussed here, as well as PSS-Biotin, a biotinylated version which can be visualized using indicator-labeled streptavidin, including streptavidin-conjugated quantum dots. Finally, a CdSe/CdS quantum dot system (PSS-Green QD) coated with the PS affinity group has also been prepared and tested as a PS sensor. These compounds are all described in the following sections.

7.2 Results and Discussion

7.2.1 PSS-480

A fluorescein-labeled analog of 3, termed PSS-480 because of the fluorescence excitation maximum, was synthesized (Dr. C. Lakshmi) and used in experiments to detect early apoptosis in a cell population treated with the chemotherapeutic agent
camptothecin. Unlike NBD, fluorescein does not exhibit an environmental polarity-dependent change in fluorescence emission intensity, so binding studies like those carried out for 1 and 3 were not possible. However, the binding affinity of **PSS-480** to PS-rich phospholipid membranes should be nearly identical to that determined for 3 since the PS binding group and the triethyleneoxy linker of each compound is completely conserved.
Figure 7.1 - Fluorescence micrographs (40X magnification) of Jurkat cells treated with camptothecin (10 µM) for 3.5 h to induce apoptosis, then stained with PSS-480 (5 µM) and 7AAD (500 ng/mL), a membrane-impermeable dye that selectively labels nucleic acids and indicates cell death. Fluorescence of cells stained with PSS-480 is shown in (a), fluorescence of cells stained with 7AAD is shown in (b), and an overlay of both (a) and (b) is shown in (c). Those cells stained only with PSS-480 are apoptotic as illustrated by exclusion of 7AAD. The apoptotic cells are indicated with circles. A phase contrast image of all cells in the field is shown in (d). No staining of healthy cells with PSS-480 was observed in the absence of treatment with camptothecin.
Jurkat cells were treated with camptothecin (10 µM) for 3.5 hours and then stained with PSS-480 and 7AAD. Necrotic cells no longer retain membrane integrity and are permeable to staining agents. The nuclear stain 7AAD was used in these staining experiments to distinguish necrotic cells from those cells in the early to middle stages of apoptosis because both populations have externalized PS. PSS-480 bound only to cells with externalized PS. If the compound only bound nonselectively to cell membranes, all cells in the population would be stained. The circled cells in Figure 7.1 are apoptotic, which can be concluded based on their staining by PSS-480 but not by 7AAD. If the cells had progressed into necrosis, they would have been stained by both compounds.

**Figure 7-2** - Phase contrast and fluorescence images (a/c and b/d, respectively) of a field of Jurkat cells treated with 10 µM PSS-480 at 0 °C (top) or 37 °C (bottom). Apoptosis was induced by treatment with camptothecin (10 µM) for 3.5 h prior to staining. (60X magnification)
Binding of PSS-480 to apoptotic cells was found to be independent of temperature or competing anions, as staining of apoptotic cells in cell culture media was performed without complications and staining at both 0 and 37 °C lead to identical visualization of apoptotic Jurkat cells (Figure 7.2). Furthermore, PSS-480 was shown to be suitable for imaging apoptosis in multiple cell lines, as shown in Figure 7.3 and Figure 7.4. Jurkat cells are nonadherent, and it was therefore critical to illustrate that PSS-480 could be used in situations where apoptosis in adherent cells was to be detected. After treating samples from the adherent Chinese Hamster Ovary and HeLa cell lines with camptothecin to induce apoptosis, staining of these cells with PSS-480 again resulted in staining only of cells with externalized PS and not of cell membranes in general.

![Figure 7.3](image)

**Figure 7.3** - Fluorescence micrographs (60X magnification) of CHO cells treated with camptothecin (10 µM) for 3.5 h to induce apoptosis, then stained with PSS-480 (15 µM) and the nuclear stain 7AAD (500 ng/mL). Fluorescence of cells stained with 7AAD is shown in (a), fluorescence of cells stained with PSS-480 is shown in (b). Phase contrast image of treated cells is shown in (c). Overlay of both (a) and (b) onto phase contrast image in (d).
Figure 7.4 - Fluorescence micrographs (60X magnification) of HeLa cells treated with camptothecin (10 µM) for 3.5 h to induce apoptosis, then stained with PSS-480 (15 µM) and the nuclear stain 7AAD (500 ng/mL). Fluorescence of cells stained with 7AAD is shown in (a), fluorescence of cells stained with PSS-480 is shown in (b). Phase contrast image of treated cells is shown in (c). Overlay of both (a) and (b) onto phase contrast image in (d).

While fluorescence microscopy provides us with an attractive visual picture of PSS-480 selectivity and its practicality for use in detecting apoptosis in actual biological research, a quantitative assessment of PS selectivity is only available from flow cytometry. The compatibility of the fluorescein fluorophore of PSS-480 with most commonly used flow cytometers is another attractive feature of this compound which makes it superior to PSS-380, which is only compatible with top-end flow cytometers equipped with UV excitation capability. When Jurkat cells treated with camptothecin to induce apoptosis were stained with either PSS-480 or Annexin V-FITC and analyzed by flow cytometry, practically identical cell populations were found to be labeled by each
reagent. The flow cytometry histograms for treated and control cells stained with PSS-480 are shown in Figure 7.5.

**Figure 7.5** - Flow cytometry histograms illustrating staining of Jurkat cells by PSS-480 and 7AAD. Horizontal axes in each panel indicate fluorescence emission intensity of the indicated dye. Control cells (top and bottom left) and treated cells (top and bottom right) exhibit similar levels of staining by 7AAD, indicating the same level of necrotic cells in the population (less than 5% in each case). Cells treated with camptothecin exhibit significantly more staining by PSS-480 than do control cells. Approximately 30% of treated cells were identified as apoptotic using PSS-480, while less than 5% of the untreated cells were stained with PSS-480.
7.2.2 PSS-Biotin

In order to develop a Zn$^{2+}$-DPA coordination compound that could be used to detect PS in a diverse range of applications in which other fluorescent probes may be used and tailored reporter elements may be necessary, a biotinylated version of the PS binding group, PSS-Biotin, was prepared (Dr. C. Lakshmi). The versatility of PSS-Biotin is limited only by the availability of the necessary labeled streptavidin. This versatility is demonstrated here, where PSS-Biotin was used to visualize apoptotic cells with multiple dye-labeled streptavidins.

To demonstrate the utility of PSS-Biotin, Jurkat cells treated with camptothecin to induce apoptosis were stained with Annexin V-FITC, 7AAD, and PSS-Biotin, followed by treatment with a blue dye-conjugated streptavidin. The fluorescent images shown in Figure 7.6 illustrate that PSS-Biotin performs just as well as a reagent for detecting cell surface PS as Annexin V. The circled cells in Figure 7.6 are stained by both PSS-Biotin/Streptavidin and Annexin V-FITC, but not by 7AAD. This indicates that these cells have externalized PS, but have not lost membrane integrity. These circled cells are apoptotic, and all the cells stained by Annexin V-FITC were also stained by PSS-Biotin/Streptavidin.

An important goal of this research was the development of a compound that could detect apoptosis in the early stages. PS externalization is detectable in apoptotic cells long before the cell membrane becomes permeable, so any reagent that could successfully detect apoptosis in the early stages should stain only the outer membrane and should not be visible in the cytosol. Figure 7.7 shows a series of cross sections taken of a single Jurkat cell stained with both Annexin V-FITC and PSS-Biotin/Streptavidin.
Figure 7.6 - Fluorescence micrographs (40X magnification) of Jurkat cells treated with 10 µM camptothecin for 3.5 h and stained with 7AAD (500 ng/mL), annexin V-FITC (BD Biosciences commercial solution), and PSS-Biotin (100 µM) with a blue-emitting streptavidin-dye conjugate (400 nM). All reagents were added simultaneously. Cells were then incubated for 15 minutes at 37 °C. (a) Cells stained with the nuclear stain 7AAD; (b) Cells stained with annexin V-FITC; (c) Cells stained with PSS-Biotin/streptavidin-Marina Blue® conjugate (460 nm emission); (d) Brightfield image of the entire field of cells. Cells in the circled regions of each image are apoptotic. No staining of healthy cells was observed in the absence of treatment with camptothecin.

The orange color on the surface of the cell is a result of the colocalization of both the green-emitting FITC and the red-emitting streptavidin-quantum dot conjugate. The fluorescence emission is restricted entirely to the periphery of the cell, indicating that neither the PSS-Biotin nor the Annexin V-FITC was able to cross the cell membrane. This observation indicates that the cell in the picture has been captured in the early stages of apoptosis and has not yet begun the late-phase processes associated with apoptosis, including membrane permeabilization.
7.2.3 Zn$^{2+}$-DPA Quantum Dot Conjugates

The promising results obtained using PSS-Biotin in combination with quantum dot-conjugated streptavidin raised the possibility of designing a single sensing reagent that did not depend on the secondary binding event between biotin and streptavidin necessary for this two-component reagent to work. Direct attachment of the Zn$^{2+}$-DPA PS-binding group to a water-soluble quantum dot presented a likely direction to take in developing a PS sensing system with the PS selectivity of the Zn$^{2+}$-DPA compounds previously described and the photophysical properties of quantum dots all in a single sensor.

Preparation of CdSe quantum dots encapsulated inside a ZnS shell (Dr. Tim Lambert, Sandia National Laboratories) was the first step to developing a workable quantum dot-based PS sensor. This CdSe/ZnS core/shell assembly was then encapsulated.
inside a phospholipid outer coating containing carboxy-polyethyleneglycol chains, rendering the assembly water-soluble. Peptide coupling of the free amine version of 3 to the carboxy groups on the surface of the quantum dot assembly produced the **PSS-QD** (Phosphatidylserine Sensor Sensor – Quantum Dot) sensor (Figure 7.8), which was used in fluorescence microscopy experiments to determine its efficacy as a reagent for detecting PS-rich cell membranes.

**Figure 7.8** – Graphical representation of the **PSS-QD** sensor for PS-rich membranes. The amine shown was coupled to the carboxy groups projecting from the surface of the quantum dot to create a multivalent PS sensor with the benefits of both the Zn$^{2+}$-DPA compounds previously described, as well as the quantum dot reporter elements commonly found on commercially available streptavidin conjugates.
**PSS-QD** performed very well as a sensor for PS-rich cell membranes and produced images very similar to those obtained using **PSS-480** and **PSS-Biotin** in fluorescence microscopy. Shown in Figure 7.9 is a population of Jurkat cells stained with both **PSS-QD** and 7AAD after treatment with camptothecin to induce apoptosis. The sensor clearly does not associate with all cell membranes, which is evidenced by the numerous cells visible in the brightfield image that are not visible in the fluorescence micrographs.

**Figure 7.9** - Fluorescence micrographs (40X magnification) of Jurkat cells treated with camptothecin (10 µM) for 3.5 h to induce apoptosis, then stained with **PSS-QD** (2.75 µM) and 7AAD (500 ng/mL). Fluorescence of cells stained with 7AAD is shown in (a), fluorescence of cells stained with **PSS-QD** is shown in (b), and an overlay of both (a) and (b) is shown in (c). Those cells stained only with **PSS-QD** are apoptotic as illustrated by exclusion of 7AAD. A phase contrast image of all cells in the field is shown in (d). No staining of healthy cells was observed in the absence of treatment with camptothecin.
Just like the other Zn$^{2+}$-DPA compounds described earlier, **PSS-QD** associates only with the cell membrane surface and does not permeate into the cytosol of apoptotic cells. Though it is possible that the mere size of the **PSS-QD** and the **PSS-Biotin**/Streptavidin PS sensors prevents their entry into the cell interior, cellular uptake of similarly sized compounds has been reported, suggesting that the Zn$^{2+}$-DPA sensors used in these experiments are not excluded from the cytosol by size alone. Figure 7.10 shows a series of cross sections taken of a single Jurkat cell treated with camptothecin to induce apoptosis and then stained with **PSS-QD**. The staining is clearly restricted to the membrane surface.

**Figure 7.10** - Cell surface labeling of a single Jurkat cell from a population treated with 10 μM camptothecin for 3.5 h to induce apoptosis. Images are 0.5 μm slices (60X magnification) taken through the cell separated by 2.5 μm. Cells were treated with 10 μM **PSS-QD**. Exclusion of **PSS-QD** from the interior of the cell indicates that only surface staining takes place.
7.3 Summary

Zn$^{2+}$-DPA coordination complexes can be developed into effective fluorescent probes for apoptosis that can be successfully used to identify apoptotic cells using both fluorescence microscopy and flow cytometry. The versatility of the system was enhanced by attaching a biotin reporter element (PSS-Biotin), making detection of apoptotic cells possible with a range of fluorescent streptavidin conjugates. The Zn$^{2+}$ coordination complexes allow users to identify apoptotic cells under Ca$^{2+}$-free conditions and with fast binding kinetics, which broadens the scope of the PS-based method for apoptosis detection. Indeed, the low molecular weight, non-protein probes presented may be adaptable to other imaging techniques, such as radiography and magnetic resonance spectroscopy. The first step toward development of such imaging reagents was achieved here by direct conjugation of a Zn$^{2+}$-DPA PS affinity group to a phospholipid-encapsulated quantum dot. This combination reagent retained the PS selectivity reported earlier for Zn$^{2+}$-DPA coordination compounds, while simultaneously providing the desirable photophysical properties associated with quantum dots. Extension of this strategy to additional reporter elements may result in sensing compounds to achieve molecular imaging of an even more diverse range of biochemical targets.
8.1 Introduction

The roles of membrane proteins in the normal physiology and biochemistry of the cell are tremendously diverse and encompass everything from ion channel activity\textsuperscript{177} to serving as receptors for key hormones and signaling molecules.\textsuperscript{178} Membrane proteins exhibit not only a variety in function, but also a diversity in structure and location in the plasma membrane (Figure 8.1). For example, there are membrane proteins that are found almost entirely inside the plasma membrane,\textsuperscript{179-184} others that are anchored into one leaflet of the bilayer by a fatty acid or other bioconjugate,\textsuperscript{185-186} and still many others that

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{membrane_proteins}
\caption{Membrane proteins occupy three distinct environments in the phospholipid bilayer. They can localize to a single leaflet of the membrane, be contained entirely within the interior of the bilayer hydrocarbon region, or span the membrane one or more times.}
\end{figure}
have alpha helices spanning the bilayer one or more times.\textsuperscript{2,177-178} While a great body of information exists on the structure and function of each type of membrane protein, it is the transmembrane proteins that will be considered in detail in the following sections. These proteins are often involved in important, well-characterized supramolecular interactions that represent a unique opportunity for chemists to contribute to our understanding of biochemical and biophysical events that take place between proteins and lipids in the cell membrane.

The transmembrane proteins fall broadly into two categories, those that span the membrane only once, and those that span the bilayer multiple times.\textsuperscript{187} This division is not arbitrary, but rather reflects the function typically associated with each class of transmembrane protein. The single-pass transmembrane proteins are often receptors for hormones and small molecule signaling compounds. They frequently have an enzymatic domain on their intracellular terminus that initiates a signaling cascade in response to ligand binding by the extracellular domain. The most well-known of these proteins may be the receptor tyrosine kinases, single-pass transmembrane proteins that dimerize and undergo transautophosphorylation upon ligand binding.\textsuperscript{178} Certain multi-pass transmembrane proteins are also important receptors, especially the G-Protein Coupled Receptors (GPCRs), which span the membrane seven times. Ion channels and ion pumps are also frequently multi-pass transmembrane proteins whose activity depends on key interactions between their alpha-helical domains spanning the bilayer.

The interactions between single-pass transmembrane proteins and between various alpha helices of multi-pass transmembrane proteins are carefully controlled and depend critically on ligand binding for normal protein activity. As a result, mutations
that introduce polar residues into the transmembrane domains of membrane protein often
cause serious problems because of the driving force such mutations present for ligand-independent transmembrane protein-protein dimerization or alteration in alpha helix interactions.\textsuperscript{45} These mutant proteins are known to cause diseases associated with
signaling cascades and ion transport, two key functions of membrane proteins.

The cystic fibrosis transmembrane conductance regulator (CFTR) provides an
illustrative example of how transmembrane protein mutations can influence protein
function. The CFTR is a 1480 amino acid protein hypothesized to span the membrane
twelve times.\textsuperscript{188} The alpha helices of the CFTR that span the membrane assemble into a
chloride channel, and failure of the protein to function properly is a known cause of
cystic fibrosis.\textsuperscript{189} Loss of Phe508 is the most common mutation which leads to poor
CFTR protein processing. Another mutation is a valine-to-aspartic acid substitution
occasionally observed at position 232 (V232D) in transmembrane four of the CFTR
protein.\textsuperscript{190-192} This non-native carboxylic acid residue forms a hydrogen bond to
 glutamine 207 in neighboring helix three,\textsuperscript{192} an interaction that attenuates the channel
activity of the protein. The D232-Q207 interaction is specifically responsible for the
decrease in activity, and a double mutant containing the aspartic acid at position 232 and
a leucine at position 207 restored the normal behavior of the protein.\textsuperscript{190} Disease-causing
interactions that can be addressed to such a precise location are attractive targets for
development of small molecules to disrupt the problematic interaction.

Another ideal model system for developing small molecules to target specific
residues in the transmembrane domains of membrane proteins is the bacterial enzyme
Diacylglycerol Kinase (DAGK) from \textit{E. coli}.\textsuperscript{193-194} DAGK is a 13 kD, 121 amino acid
membrane protein with three transmembrane domains. The protein is among the most widely studied of all membrane proteins, and is amenable to a wide variety of experimental conditions, including enzyme kinetic measurements,\textsuperscript{195-196} NMR spectroscopy,\textsuperscript{197-198} and other biophysical perturbations.\textsuperscript{199-201} The active form of DAGK is a trimer that forms in the membrane,\textsuperscript{195} bringing together key residues from each monomer to form active sites at each protein-protein interface.\textsuperscript{196} DAGK trimerization is mediated at least in part by glutamic acid 69 in transmembrane two of the protein, and treatment of DAGK with a truncated version corresponding to only the transmembrane two sequence resulted in a complete loss of DAGK activity.\textsuperscript{195} The Glu-69 side chain is a site that could be targeted by designed molecules in order to inhibit the trimerization necessary for enzyme activity and illustrate the utility of small molecules for modulating membrane protein function.

The following sections describe the effects of rationally designed molecules on membrane protein structure and activity. Using the membrane protein systems described, CFTR and DAGK, rationally designed molecules have been shown to interact with membrane proteins and modulate both their extent of oligomerization (CFTR) as well as biochemical activity (DAGK).

8.2 Results and Discussion

8.2.1 Disruption of CFTR Aggregation by Designed Molecules

The V232D mutation observed in transmembrane domain four of the CFTR protein is a reported cause of certain forms of cystic fibrosis, and therefore represents an attractive model system for use in developing small molecules that can interact with polar
residues in a membrane environment and inhibit unwanted protein interactions in a membrane. A truncated version of CFTR corresponding only to the transmembrane four sequence has been shown to be a representative model for the larger CFTR protein.\textsuperscript{191} By working with this truncated segment, it was possible to employ electrophoresis as a screening technique for evaluating small molecules designed to interact with the mutant protein in a membrane-like environment.

The transmembrane four sequence of CFTR, residues 219-242, \((\text{LQASAFCGFLIV}^{232}\text{LALFQ}^{237}\text{AGLGR})\) has a cysteine at position 225 which oxidizes to form a disulfide-linked dimer. The mutant form of this dimer then oligomerizes into higher-order assemblies, especially tetramers and octamers containing, respectively, two and four dimers, by hydrogen bonds that form between the mutant aspartic acid at position 232 and the wild-type glutamine at position 237. This oligomerization is not observed in a transmembrane four sequence containing only the wild-type valine at position 232, indicating clearly that the observed oligomerization is a result of the carboxylic acid of the mutant sequence (Figure 8.2).

Transmembrane proteins normally require a membrane or a micelle environment in order to fold and function properly. For this reason, small molecules with the same amphiphilic properties as phospholipids were initially chosen as candidates for interaction with the polar aspartic acid side chain in the CFTR mutant model. As a starting point, thymine-derived amphiphiles 20 - 22 were prepared as described by Nowick \textit{et al.}\textsuperscript{202} with alkyl chains of varying lengths and tested by electrophoresis to determine the extent to which they bound the CFTR mutant transmembrane four sequence. The C-4 and C-8 analogs were previously prepared by Nowick \textit{et al.}, and the
C-12 variant was synthesized according to the analogous procedure. The quaternary ammonium headgroup of these compounds was expected to reside primarily in the polar headgroup region of a micelle, while the pendant thymine group would partition into the apolar hydrocarbon region of the micelle and present its hydrogen bonding functionalities for interaction with the carboxylic acid side chain of the D232 or Q237 residue in the mutant CFTR transmembrane four sequence.
Figure 8.2 – (TOP) Hydrogen bonding-mediated oligomerization of the disulfide-linked CFTR transmembrane 4. The Asp-Gln hydrogen bonding results in formation of protein tetramers, hexamers, and oligomers in the environment of a micelle. (BOTTOM) Possible mechanism whereby the thymine-alkylammonium compound 22 disrupts the hydrogen bond-mediated oligomerization of CFTR transmembrane 4 in a micelle.
Peptide sequences corresponding to both the wild type and the V232D mutant forms of CFTR transmembrane 4 were prepared by traditional solid phase peptide synthesis (Ed O’Neil). An aqueous solution (2 mg/mL) of each peptide was subjected to air oxidation for approximately ten days in order to allow the disulfide linkage to form between monomers. Even after ten days, the extent of peptide dimerization reached only around 50%, and longer periods did not result in substantially more dimer formation. Treatment of the peptide with oxidizing agents, including hydrogen peroxide, iodine, bromine, and hypochlorite, only resulted in peptide degradation and no detectable formation of the peptide dimer. For this reason, the transmembrane 4 solution in which dimers were formed by air oxidation was used as the stock solution for experiments where 20-22, as well as all other subsequent derivatives, were tested to determine their effect on mutant CFTR transmembrane 4 oligomerization. Each compound was added to a 10 µL aqueous sample of the mutant peptide (2 µg, 118 µM) such that the compound : peptide ratio was 127:1. After a thirty minute incubation at room temperature, the sample was loaded and run on an SDS-PAGE gel (4-12% gradient) (Figure 8.3). An intense band corresponding to the molecular weight of a transmembrane 4 peptide octamer is clearly visible in the lanes containing compounds 20 and 21, while the same band in the lane containing 22 is much more diffuse. (For a control lane containing no added molecule, see Figure 8.4.) Furthermore, 22 induced a distortion of the lower-order bands and caused the peptide to concentrate more in the band corresponding to dimer, meaning that aggregation of the disulfide-linked dimers had been disrupted by 22. In order to determine the extent to which 22 was able to effect such a disruption of the hydrogen bonding-mediated oligomerization, a titration experiment was performed, where
increasing amounts of 22 were added to the CFTR transmembrane 4 peptide and run on an SDS-PAGE gel (Figure 8.4).

![Image of a gel with molecular weight markers and protein bands labeled as monomer, dimer, tetramer, hexamer, and octamer.]

**Figure 8.3** – CFTR transmembrane 4 peptide (2 µg/lane) run on a gradient (4 to 12%) SDS-PAGE gel. Lane A contains standard molecular weight markers. Lane B contains wild type transmembrane 4 peptide. Lanes C-E contain V232D mutant transmembrane 4 peptide in the presence of 127 equivalents of 20, 21, and 22, respectively.

The results indicate a concentration-dependent effect of 22 on the extent of transmembrane 4 peptide oligomerization. The intensity of the octamer band begins to visibly decrease at a peptide-to-compound ratio of 1:64. At the same ratio, the combined monomer-dimer band, presumably now containing more dimers than when lower amounts of compound were present, also begins to exhibit the characteristic waviness observed in the experiment depicted in Figure 8.3. Both effects become more
pronounced at increasing peptide-to-compound ratios, finally reaching the point where those bands at aggregation states below the level of octamer nearly smear into a single nondiscernable band when the peptide-to-compound ratio reaches 1:127.

The hydrogen bonding properties of the thymine functionality in 22 allow a number of interactions with the transmembrane 4 peptide to be possible, and simultaneously offer an explanation as to why 20 and 21 were ineffective at causing a change in the oligomerization state of the peptide. In the electrophoresis experiment, the transmembrane 4 peptide oligomer is presumably incorporated into an SDS micelle. When 22 partitions into the micelle, the thymine group must be able to reach down into the hydrophobic environment of the micelle interior in order to interact with the carboxylic acid moiety of the aspartic acid at position 232 or the glutamine primary amide at position 237 in order to affect the hydrogen bonding-mediated oligomerization of the peptide. In 22, the twelve-carbon linker separating the quaternary ammonium headgroup and the pendant thymine is perhaps just long enough for the thymine to reach the micelle interior while leaving the ammonium cation to interact with the anionic SDS headgroups. In 20 and 21, the shorter alkyl linkers likely prohibit the thymine from partitioning to a sufficient depth to allow it to make contact with the key side chains that mediate peptide oligomerization.

Hydrogen bonding between the carboxylic acid side chain at position 232 and the primary amide at position 237 of the mutant peptide is the driving force for protein oligomerization, and hydrogen bonding capabilities are presumably necessary for any small molecule to be able to affect this process. The thymine of 22 can act as both a hydrogen bond donor and acceptor. The imide proton of the thymine is particularly
acidic, making 22 a strong hydrogen bond donor. In order to prove that the dual roles of both a hydrogen bond donor and acceptor are necessary for a compound to interact with the transmembrane four peptide and disrupt oligomerization, compound 23 was prepared and tested in the same way as 22. The N-methyl compound 23 could be synthesized in near quantitative yield by treatment of 22 with potassium carbonate in the presence of methyl iodide while stirring at room temperature for 96 h. Additionally, 23 could be made from the common thymine-derived primary alkyl bromide 22a used in preparation of 22 (See synthesis – Chapter 10). Since 23 can act only as a hydrogen bond acceptor, it was expected to have no real effect on the oligomerization state of the CFTR transmembrane four peptide. Figure 8.5 shows the effect of 23 on the transmembrane four peptide when tested in the same SDS-PAGE electrophoresis assay described earlier.

Figure 8.4 – CFTR transmembrane 4 peptide (2 µg/lane) run on a gradient (4 to 12%) SDS-PAGE gel. Lane A contains wild type transmembrane four peptide. Lanes B-H contain V232D mutant transmembrane four peptide in the presence of 0, 21, 42, 64, 85, 106, and 127 equivalents, respectively of 22.
Figure 8.5 – CFTR transmembrane 4 peptide (2 µg/lane) run on a gradient (4 to 12%) SDS-PAGE gel. Lane A contains wild type transmembrane four peptide. Lanes B-H contain V232D mutant transmembrane four peptide in the presence of 0, 21, 42, 64, 85, 106, and 127 equivalents, respectively, of 23.

While the waviness apparent in the bands observed in Figures 8.3 and 8.4 is not obvious here, compound 23 did cause an observable concentration-dependent shift of each band to an apparently smaller molecular weight. The intensity of the octamer band in Figure 8.4 also does not decrease as dramatically as it does in the case of compound 22. These
observations taken together suggest that 23 does not interact with the transmembrane four peptide in the same way as 22, but that it does have at least some effect on either the structure or the environment of the peptide during the electrophoresis experiment.

The properties of the headgroup of thymine-derived compounds like 22 were also found to be important for controlling the interaction of these molecules with the transmembrane four peptide. The tetraalkylammonium cationic headgroup of 22 is a stabilizing feature of the compound in the anionic environment of the SDS micelle surface. The ammonium group of 22 likely anchors the compound into the micelle by forming charge-charge interactions with the anionic SDS headgroups, while the thymine partitions into the alkyl interior of the micelle where it can interact with the protein side chains and exert an effect on peptide oligomerization. Two compounds with different headgroup properties were prepared, 24, with an anionic sulfur oxyanion headgroup, and 25, with the neutral, though still polar, hydroxyl headgroup. Both 24 and 25 were tested
in the same fashion as 22, where the CFTR transmembrane four peptide was treated with increasing concentrations of each molecule in order to determine the effect each compound would have on peptide oligomerization. In each case, no effect on peptide oligomerization was detectable up to a peptide:compound ratio of 1:127. (Figure 8.6 for 24 and Figure 8.7 for 25) Several explanations can be made for these observations, but the most likely is the failure of both 24 and 25 to partition into the anionic SDS micelle surrounding the CFTR transmembrane four peptide. In the case of the successful compound 22, the favorable electrostatic interaction that could be formed between the ammonium headgroup of 22 and the anionic headgroup of SDS molecules presented a significant driving force incorporation of the compound into the micelle. In the subsequent cases, particularly that of 24, where the headgroup is not favorably attracted, and is in fact repelled by the matched anionic electrostatic forces, the compounds may well remain simply suspended in the aqueous environment surrounding the micelles.

Figure 8.6 – CFTR transmembrane 4 peptide (2 µg/lane) run on a gradient (4 to 12%) SDS-PAGE gel. Lane A contains wild type transmembrane four peptide. Lanes B-H contain V232D mutant transmembrane four peptide in the presence of 0, 21, 42, 64, 85, 106, and 127 equivalents, respectively, of 24.
Each of the tested compounds is water soluble, and without the favorable electrostatic interactions between headgroups to provide a driving force for partitioning of the tested compound into the micelle, it is likely that 24 and 25 failed simply because the thymine functionality was never placed in the necessary environment to form hydrogen bonds with the amino acid side chains of the CFTR transmembrane four peptide.

**Figure 8.7** – CFTR transmembrane 4 peptide (2 µg/lane) run on a gradient (4 to 12%) SDS-PAGE gel. Lane A contains wild type transmembrane four peptide. Lanes B-H contain V232D mutant transmembrane four peptide in the presence of 0, 21, 42, 64, 85, 106, and 127 equivalents, respectively, of 25.

Of the compounds tested, only 22 was found to be successful at disrupting CFTR transmembrane four aggregation to any appreciable extent. The combination of hydrogen bond donor and acceptor properties of 22, along with its alkyl chain that presumably provides an appropriate match with the SDS surfactant that forms the micelles encapsulating the peptide, gave 22 the attributes required to interact with the transmembrane four peptide in the way required to break up hydrogen bond-mediated oligomerization. This point is illustrated no more clearly than in a head-to-head comparison of 22 and 23, which suggests that the N-methylated derivative 23 does not perform at the same level as its parent compound 22 (Figure 8.8). This observation lends
support to the initial hypothesis that an appropriate hydrogen bonding partner is required for disruption of the hydrogen bonding-mediated oligomerization of the CFTR transmembrane domain 4, but additional studies are needed.

Figure 8.8 – CFTR transmembrane 4 peptide (2 µg/lane) run on a gradient (4 to 12%) SDS-PAGE gel. Lane A contains wild type transmembrane four peptide. Lanes B-D contain V232D mutant transmembrane four peptide in the presence of no added compound (B), or 127 equivalents of 22 (C) or 23 (D).

8.2.2 Inhibition of Diacylglycerol Kinase by Phospholipid-Derived Ureas

The active form of DAGK is a trimer that forms in the membrane stabilized by a network of hydrogen bonding interactions (Figure 8.9). Three active sites form in each trimer, one at each interface of transmembrane domain two. Because trimerization is a prerequisite for enzyme activity, inhibition of the hydrogen bonding interactions that hold the trimer together should result in inhibition of enzyme activity. Hydrogen bonding between DAGK monomers is increasingly favored by the environment where the interactions must form, the interior of a membrane bilayer or micelle. Because of the
apolar nature of this environment, the propensity for hydrogen bonding is greater, meaning that the presence of alternative hydrogen bonding partners in the membrane or micelle interior would likely result in at least some degree of disruption of the DAGK trimerization required for complete enzymatic activity.

Since the enzyme inhabits a membrane environment, a logical inhibitor to pursue was a phospholipid-based compound capable of presenting a hydrogen bonding partner in the apolar interior of a membrane or micelle. Using a previously described phosphatidylcholine compound\textsuperscript{203} with a primary amine at the terminal end of the acyl chain in the 2-position, a class of phospholipid ureas was prepared (Ed O’Neil) and tested to determine the extent to which they could inhibit DAGK activity. The potential inhibitors were tested using a known coupled enzyme assay (Figure 8.10) based on the UV absorbance of NADH. \textsuperscript{195-196} In this assay, the more quickly DAGK converts its substrate to product, the more quickly the UV absorbance of NADH in the assay mixture disappears. The phosphatidylcholine-derived compounds tested in the DAGK inhibition assay each had a urea at the terminus of the acyl chain in the 2-position. The ureas were readily prepared by reaction of the known primary amine with the corresponding isocyanate, a reaction that was initially performed to yield the phosphatidylcholine ureas (PC-ureas) \textsuperscript{26} and \textsuperscript{27}, where the para position of each phenylurea was substituted with a nitro or a \textit{t}-butyl group, respectively. The PC-ureas were initially chosen as potential compounds for inhibition of DAGK because of the expected incorporation of these compounds into the phospholipid environment of the vesicle or micelle in which the enzyme would exist, as well as the known binding affinity of ureas for carboxylates.\textsuperscript{204}
Figure 8.9 – Trimerization of DAGK leads to formation of the active enzyme complex. Three active sites form in the trimer, one at each of the interfaces of transmembrane domain two of the constituent monomers.

Figure 8.10 – Coupled enzyme assay used to evaluate potential inhibitors of DAGK.
Both 26 and 27 were tested in the coupled assay shown in Figure 8.9 to determine their effect on DAGK activity. In each case, the time required for NADH absorbance at 340 nm to decrease by a factor of two was extended (Figure 8.11), by approximately a factor of five in the case of 26 and by nearly a factor of four for 27. Though the extent of DAGK inhibition by 26 and 27 was the same within experimental error, the more strongly electron withdrawing nitro group at the para position of 26 would be expected to make 26 a more potent inhibitor simply because of the increased acidity of the corresponding urea proton and the subsequent enhancement in hydrogen bonding.
tendency this would impart on 26 relative to 27, which is substituted with the electron donating t-butyl group.

![Graph](image)

**Figure 8.11** – Normalized UV absorbance of NADH at 340 nm (A/A_0) when DAGK (10 µg) was treated in the coupled assay described with either no inhibitor (far left curve), compound 26 (far right curve), or compound 27 (center curve) at a ratio of 10:1 (w/w). These data are representative experiments performed at 25 °C in a 60 mM PIPES buffer solution at pH 6.85.

Both 26 and 27 presumably exert their inhibitory effects by binding to either the carboxylate of glutamate 69 in transmembrane 2, which has been postulated to mediate the enzyme trimerization required for activity, or to one of the other residues that form the active site in the bound trimer. To prove that hydrogen bonding to one or more functionalities on the protein was a necessary prerequisite to inhibitory activity, the Fmoc-protected amine 26a, a precursor to 26 and 27, was added to the enzyme assay at the same levels as 26 and 27 and tested for DAGK inhibition. As expected, the activity of DAGK was decreased only slightly, by only approximately a factor of two. This likely reflects a change in the micelle environment or a steric constraint imposed by the
presence of the bulky Fmoc group in the micelle interior and not a true inhibition of the enzyme by interaction with the compound. This conclusion is supported by the observation that similar inhibitory activity was observed when POPC was added to the enzyme assay at the same level as the Fmoc-protected amine. POPC clearly has no capacity for interacting with DAGK in any hydrogen bonding fashion, and the presence of POPC, and also the Fmoc-protected amine of PC, in the octyl-\(\beta\)-D-glucopyranoside micelles used in the assay is likely responsible for the observed slight decrease in enzyme activity. A complete summary of inhibition values obtained for 26 and 27, as well as the control compounds described, is presented in Table 8.1 and Figure 8.12.

**TABLE 8.1**

<table>
<thead>
<tr>
<th>Additive</th>
<th>(t_{1/2}) for NADH Absorbance Decrease (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additive</td>
<td>6.04 ± 1.45</td>
</tr>
<tr>
<td>26</td>
<td>30.77 ± 13.91</td>
</tr>
<tr>
<td>27</td>
<td>22.10 ± 7.47</td>
</tr>
<tr>
<td>26a</td>
<td>13.00 ± 6.79</td>
</tr>
<tr>
<td>POPC</td>
<td>10.05 ± 2.62</td>
</tr>
</tbody>
</table>

**NOTE:** Values shown are averages of three separate experiments done using the coupled enzyme assay based on NADH UV/Vis absorbance. Values represent the average time required for the value of \(A_{340\,\text{nm}}\) to decrease by a factor of two.
The product of dioleoylglycerol phosphorylation by DAGK is the naturally occurring compound phosphatidic acid, which also inhibited the activity of DAGK when added to the beginning reaction mixture in a 10:1 ratio (w/w) with DAGK. The inhibitory effect of PA at this ratio, approximately a five-fold inhibition, was similar to that observed for the nitro-substituted compound 26, though PA, like any other compound that exhibits product inhibition, likely binds in the active site of DAGK and does not exert its inhibitory effect by disrupting enzyme trimerization in the micelle. An
important note should be made about the reproducibility of the inhibition results presented here. All data presented in this section represents the results of no less than three separate experiments, and all results have been reproduced at least as many times, but the coupled enzyme assay used here suffers from considerable variability. Those who may wish to apply this technique to other research problems, or reproduce the results presented here, must do so with the understanding that outlying results are commonplace and that many repetitions of the same experiment must often be carried out in order to obtain a data set with reasonable accuracy.

As a final test to ensure that the PC-urea molecules 26 and 27 did actually interact with the DAGK protein and did not exert their inhibitory effect via some other unknown mechanism, fluorescence quenching experiments were performed using a third PC-urea synthesized specifically for these experiments, compound 28. DAGK fortunately contains three tryptophan residues which fluoresce when excited at 280 nm. Interaction of 28 with DAGK was expected to result in a quenching effect mediated by the bromine at the terminal end of the molecule. It was necessary to depart from the phenylurea design used in 26 and 27 in order to develop a bromine-containing PC-urea to use in these quenching experiments. When a para-bromophenyl-PC-urea was used instead of 28, a photochemical decomposition product was formed that resulted in inconclusive, nonspecific changes in the fluorescence emission spectra of the DAGK solution. By switching from the aryl bromide to the alkyl bromide of 28, this photodecomposition was eliminated.
When an aqueous solution of DAGK (10 µg/mL) was titrated with an aqueous solution of 28, a concentration-dependent quenching of fluorescence intensity was observed when monitored at 350 nm. During the course of the titration, not only does the fluorescence emission intensity decrease, but the emission maximum is shifted to progressively higher wavelengths, from approximately 310 nm at the beginning of the titration to around 365 nm when saturated with 28 (Figure 8.13). A genuine criticism of this experiment is the fact that it was performed in aqueous solution and not in the micelle environment used for the enzyme inhibition experiments discussed earlier. It could be argued that in the absence of micelles or vesicles, 28, or any other PC-urea, would be driven to associate with DAGK by simple hydrophobic interactions, leading to a tryptophan fluorescence quenching that is independent of any specific interactions. This argument is negated by the observation of no change in the fluorescence emission spectra of DAGK when titrated with 29, a PC derivative where 12-bromododecanoic acid has been esterified at the sn-2 position, which would be expected to result in the same level of tryptophan fluorescence quenching if hydrophobic interactions were responsible for the quenching observed with 28. These data indicated the formation of a tight
interaction between the PC-urea compounds and the DAGK protein molecules. The rational for performing the experiments in water and not in a micelle environment is one of practicality. When incorporated into micelles, the fluorescence emission spectrum of DAGK becomes so broad that it is impossible to discern any genuine changes in either intensity or emission maximum during a titration.

![Chemical Structure](image)

**Figure 8.13** – Fluorescence emission spectra ($\lambda_{exc.} = 280$ nm) of an aqueous solution of DAGK (10 µg/mL) when titrated with 28 up to an ending concentration of 30 µg/mL.
8.3 Summary

Small molecules can be used to influence membrane protein function and oligomerization. By exploiting of the unique apolar environment of the bilayer interior in which membrane proteins are found, it is possible to take advantage of hydrogen bonding interactions to target specific chemical functionalities present in transmembrane proteins. Often these residues are present as a result of an aberrant mutation that leads to unwanted protein activity, and in other situations the mutation causes a protein not to function. In this chapter, examples have been provided to illustrate that small molecules can exert a targeted effect on a membrane protein. Both the CFTR transmembrane domain 4 and the enzyme DAGK were shown to be affected by small molecules designed to present a specific hydrogen bonding partner to a key residue, but both systems suffer from technical challenges that will require more work in these areas. For example, in the CFTR system described first, no tested technique was able to cause complete oxidation of the transmembrane domain 4 monomers to disulfide-linked dimers. The presence of free monomer likely had some effect on the outcome of the gel electrophoresis experiments carried out as a part of this work, but the type and magnitude of such an effect remain unknown. The DAGK enzyme inhibition experiments were dependent on a coupled assay involving eleven different components, and reproducibility of the results of these experiments remains a problem. Though the data presented in this chapter has been reproduced and validated, use of this coupled assay requires considerable commitments of time and resources, a consideration that must not be overlooked by others who may wish to apply this assay to their own research. Though neither example represents an exhaustive study of how the compounds described affect their target proteins, each case
has laid the groundwork for future experiments in this area that will potentially lead to significant developments in our understanding of how to modulate the important cellular processes controlled by proteins in the cell membrane.
CHAPTER 9

SUMMARY OF FINDINGS, DIRECTIONS FOR FUTURE STUDIES, AND
CONCLUDING STATEMENTS

9.1 General findings

The principle purpose of the research described in this dissertation has been to illustrate that the complex biological processes that take place in the dynamic environment of the phospholipid membrane surrounding eukaryotic cells can be both monitored and controlled by small molecules. Nature has chosen to regulate membrane processes primarily through the evolution of proteins with tailored functional properties, including the capacity for selective recognition of certain membrane components. By closely examining the mechanism by which these proteins carry out the remarkable molecular recognition events that control cell biochemistry, it is possible to design small molecules with similar properties that can participate in these same binding events, but without the unfavorable properties of proteinaceous reagents. From the experiments described in the preceding chapters, two important conclusions can be drawn. First, the proteins evolved by nature to carry out specific molecular recognition events in the matrix of the cell membrane can be effectively replaced by small molecules that have the same critical chemical properties as the protein. This point was illustrated clearly by the observation that PSS-480 and PSS-biotin, as well as the ligand-conjugated reagent PSS-
quantum dot, could be used as detection agents for apoptotic cells just as effectively as the much larger protein Annexin V. Secondly, by utilizing appropriate chemical interactions in the design of a small molecule, it is possible to engineer a compound to interact with a biological binding partner in a specified region of the membrane. The amphiphilic compounds described in Chapter 8 illustrate this concept. By taking advantage of the driving force for membrane partitioning offered by hydrophobicity, it was possible to design a molecule that would position a hydrogen bonding species in the correct environment for interaction with a chemical functionality commonly understood to be found in the apolar hydrocarbon region of the bilayer interior. While important findings in their own right, extension of these principles to even more ambitious areas of research may lead to key advances in areas of human health and disease mitigation. More detailed descriptions of the findings from the research described here are presented in the following sections, along with possible opportunities that exist for application of this work to yet unsolved problems of molecular recognition in a biological environment.

9.2 Key Findings and Important Lessons Learned About Membrane Surface Recognition

9.2.1 PS recognition by the “Three-Component-Assembly” Process

Nature has developed an intricate process for controlling certain protein-membrane binding interactions. The use of divalent metal cations, particularly Ca$^{2+}$, to bridge an anionic membrane surface and a protein is prevalent in normal cellular processes. The metal-mediated binding of the PS-recognition protein Annexin V to anionic membranes was the basis for development of the Zn$^{2+}$-DPA coordination
compounds presented in Chapters 3-6. Just like the Annexin V protein, these DPA conjugates can only bind PS-rich membranes in the presence of a bridging divalent metal ion, Ca$^{2+}$ for Annexin V and Zn$^{2+}$ for the DPA conjugates described earlier. This three-component-assembly process helps guarantee the specificity of each compound for membranes with a net anionic charge. A single anionic species in a healthy membrane surface does not present a sufficiently strong driving force for divalent metal cation accumulation, and therefore is not recognized by the sensing species that require the three-component-assembly process for binding. This same paradigm ensures that the sensing compounds do not associate with anionic species floating freely in solution. Without the electrostatic character of the anionic membrane surface, there is not a sufficient buildup of divalent metal cations to facilitate recognition of monodisperse phospholipids not sequestered in a membrane environment.

9.2.2 Multivalency Does Not Appear to be a Factor in Anionic Membrane Recognition

Despite the apparent multivalency of Annexin V, which has four canonical PS-binding units per protein molecule, and the implications for membrane binding this structure conveys, Zn$^{2+}$-DPA coordination compounds could not be made to bind the membrane more tightly by the addition of extra Zn$^{2+}$ binding units. The obvious lack of a multivalency effect described in Chapter 5 is an interesting observation, and though it cannot readily be rationalized based on the available data, several speculative hypotheses can be suggested. The most plausible of these is the occurrence of entropy-enthalpy compensation, where the added favorable enthalpy of binding associated with multivalent Zn$^{2+}$-DPA coordination complexes is offset by disfavored entropy.
resulting from rigidification of the complex or lateral ordering of phospholipids in the membrane monolayer induced by Zn\(^{2+}\)-DPA coordination complex binding. All efforts to prove the occurrence of an entropy-enthalpy compensation effect by calorimetry were inconclusive, but the known ordering of phospholipids caused by Annexin V binding suggests that such an effect is possible, if not probable.

9.2.3 Zn\(^{2+}\)-DPA Coordination Complexes can be Used to Identify Apoptotic Cells

The selectivity of the Zn\(^{2+}\)-DPA coordination complexes described in the preceding chapters for anionic phospholipids made them ideal candidates for detecting apoptosis in eukaryotic cells, where the surface of the cell membrane becomes enriched in the anionic phospholipid PS in the early stages of apoptosis. Development of Zn\(^{2+}\)-DPA coordination complexes into the successful sensors PSS-480 and PSS-Biotin resulted in compounds that can perform equally as well as Annexin V-FITC in head-to-head comparisons in assays for detection of early apoptosis. Moreover, the Zn\(^{2+}\)-DPA coordination complexes are all localized to the cell surface of apoptotic cells and do not cross the plasma membrane into the cytosol. This is an important indicator of where in the process of apoptosis the cell is found when stained with either PSS-480 or PSS-Biotin. If the compounds permeated the membrane, it would be difficult to distinguish apoptotic cells from cells that have become necrotic. In both cases the cell surface is enriched with PS, but when cells become necrotic they no longer maintain membrane integrity and significant membrane permeabilization ensues. Since PSS-480 and PSS-Biotin do not penetrate the cell membrane, it is possible to distinguish apoptosis from necrosis by observing staining only of the cell surface.
9.3 Directions for Future Research Involving Anionic Membrane Surface Recognition

9.3.1 Targeting Other Anionic Phospholipids Present in Lower Fractions

The surface of eukaryotic cells becomes enriched with anionic PS upon initiation of apoptosis, and when the membrane asymmetry is eventually lost completely, the outer membrane leaflet may contain between 5-10% PS. Though 5% is a small fraction of the total, it is still substantially higher than the levels of certain other anionic species found in the membrane, notably the phosphoinositides. Certain of these compounds, especially IP$_3$ and PIP$_2$, are important regulators of key signal transduction cascades and control many events associated with cell growth and disease. Though their relatively high anionic charge density makes them attractive targets for molecular recognition by cationic species, their very low abundance makes them a more formidable target for sensing than first appearances may suggest. Because of their important role in cellular signal transduction cascades, the levels of free PIP$_2$ and IP$_3$ in the membrane may be as low as $\leq$1%. Developing Zn$^{2+}$-DPA coordination complexes to recognize these membrane components would be a step forward in terms of both specificity and sensitivity of molecular recognition systems since a successful compound would be required to report the presence of the target compounds even in the presence of other anionic species, including PS, and recognize the very low levels of analyte present. One hypothesis for a PIP$_2$ or IP$_3$ sensing compound based on the Zn$^{2+}$-DPA coordination complex strategy is incorporation of Zn$^{2+}$-DPA groups into a more compact environment to facilitate binding to the more densely packed anionic charge of PIP$_2$ or IP$_3$ in preference to the more commonly encountered anion of PS. Initial studies using the Zn$^{2+}$-DPA coordination complexes in hand would undoubtedly have to be carried out in
advance of any new synthetic efforts to determine the extent to which PIP$_2$ and IP$_3$ are bound by Zn$^{2+}$-DPA coordination complexes, and with that knowledge, new compounds could be prepared with properties more suited to PIP$_2$ and IP$_3$ recognition.

9.3.2 Using More Biologically Available Divalent Cations as the Bridging Metal

One potential drawback to use of the Zn$^{2+}$-DPA coordination complexes described in the preceding sections, particularly those without the phenoxide anion, is the dissociation of one or both Zn$^{2+}$ cations from the organic scaffold. Though the three-component assembly process that makes these compounds successful sensors for PS rich membranes depends on dissociation of at least one Zn$^{2+}$, the applicability of this model, while successful in vitro, to in vivo systems remains in question. The levels of free Zn$^{2+}$ in the human bloodstream are very low, and any dissociation of Zn$^{2+}$ from the Zn$^{2+}$-DPA coordination complexes in the body means that the dissociated Zn$^{2+}$ ion(s) can likely be bound by some other coordinating ligand. This is unlike the situation with Ca$^{2+}$, where levels of free Ca$^{2+}$ can be as high as 1-2 mM. A PS sensing system based on Ca$^{2+}$ coordination would therefore likely be a promising strategy for in vivo detection of PS using the three-component assembly process already shown to be successful. Such a model is already at work in biology, both in the Annexin V protein used as the model on which much of this work was based, and the C2 domains commonly found in many phospholipases and other membrane binding proteins. By examining such Ca$^{2+}$-binding proteins and using information about the mechanism by which they are bridged to the membrane surface, it may be possible to develop a PS sensing compound more akin to
those used by nature and bound to the anionic membrane surface through the more abundant Ca$^{2+}$ ions present in the blood.

9.3.3 *In vivo* Applications of Anionic Membrane Surface Recognition

The ultimate extension of the work described in the preceding chapters is the *in vivo* visualization of apoptotic cells, and even apoptotic tissue, during the course of chemotherapeutic treatment. As the medical community moves ever closer to an operational system of individualized medicine, the need for physicians to quickly assess the suitability of a treatment will continue to grow. By continuing to refine the Zn$^{2+}$-DPA coordination complexes described earlier into reagents that can be used to monitor the onset of apoptosis, future researchers may be able to extend these studies into discoveries that can make genuine impacts on the practice of medicine. The modularity of the “affinity group-linker-reporter element” approach employed in the design of the Zn$^{2+}$-DPA coordination complexes described in this work is readily amenable to incorporation of groups already in use in medical imaging, including $^{19}$F, and by installing the correct chelating moiety, reporter elements for magnetic resonance imaging or radioimaging could be installed in order to make the Zn$^{2+}$-DPA coordination complexes immediately applicable in existing biomedical applications. Development of this technology using the Zn$^{2+}$-DPA coordination complexes presented here could be an important step toward determining the efficacy of treatment strategies at the earliest of stages.
9.3.4 Non-Imaging Applications of Anionic Membrane Surface Recognition

Beyond the need to detect the presence of anionic membranes in the body in order to monitor cell death and apoptosis visually, recognition of cell membranes rich in anionic phospholipids may be applicable to other biological processes important not only from the perspective of molecular recognition, but also from the point of view of physicians providing care to patients. Phagocytosis of apoptotic cells, for example, is a key process that normally takes place as the body addresses in its own way the myriad problems that arise and go unnoticed in all healthy individuals. A PS receptor on the surface of macrophages is known to be involved to some degree in the process of phagocytosis. One could easily imagine how the Zn\textsuperscript{2+}-DPA coordination complexes described throughout the preceding chapters may be used to modulate the process of phagocytosis, helping to either initiate the process or slow the process down, depending on the identity of the Zn\textsuperscript{2+}-DPA coordination complex chosen. For example, it may be possible, using the unlabeled PS receptors described in Chapter 6, to cover the surface of a cell with exposed PS and shield the cell from the PS receptors on the macrophage, therefore inhibiting phagocytosis. Conversely, by using PSS-Biotin and anti-biotin antibodies, it would be potentially possible to coat the surface of an apoptotic cell with antibodies in a process similar to opsonization, thereby facilitating the uptake of the apoptotic cell by macrophage through phagocytosis. Both possibilities are open questions that may merit consideration by future researchers.
9.4 Directions for Future Research Involving Recognition of Polar Residues in Transmembrane Proteins

The disruption of CFTR transmembrane four aggregation and the inhibition of DAGK activity discussed in Chapter 8 both illustrate that small molecules have a place in controlling the activity of membrane proteins through the molecular recognition events that allow compounds to selectively target polar residues of transmembrane proteins in the hydrocarbon region of a membrane bilayer. Though the examples of proteins used in this work to illustrate the feasibility of membrane protein control using small molecules were single-pass transmembrane proteins, there is no reason to believe the technology described cannot be extended to the more complex multi-pass transmembrane proteins, including ion channels and the seven transmembrane domain proteins. These proteins control such a variety of key cellular processes that it is difficult to single out any disease or malfunction that merits attention more than the others. By expanding the ideas developed in Chapter 8 and creating molecules that can modulate the activity of membrane proteins, future researchers can tackle some of the most vexing problems of modern medicine. Though accurate reproducibility of the DAGK inhibition data presented in Chapter 8 remains a problem, the observation of some effect of the inhibitors described on DAGK activity is unmistakable. The experiments and the data discussed in Chapter 8 offer an important starting point for future researchers to build upon in search of compounds to control membrane proteins and restore function to inoperable cellular machinery.
9.5 Concluding Remarks

The complex chemical properties of the eukaryotic cell membrane make selective recognition of any single membrane component a challenging goal. Considering the extracellular glycosylations that decorate the membrane surface, the transmembrane proteins that protrude through the membrane and hang from the bilayer, as well as the phospholipids themselves, it is not surprising that few examples of molecular recognition processes at a cell membrane surface using designed small molecules populate the literature. It has been shown throughout this work that despite the complexity of the chemical environment of the cell membrane, it is not beyond the reach of chemical design. By capitalizing on the forces used by nature in its own design of membrane binding compounds, it is possible to engineer molecular sensors to selectively recognize one component of a cell membrane while simultaneously ignoring the many, many other similar species in the same vicinity as the target. Possibilities have been presented for expanding and extending the findings described here to even greater problems facing chemistry, biology, and medicine. While the applications for which the compounds presented in this work were developed may raise new possibilities for the medical community of the future, it may well be the application of these compounds, and similar chemical species based on these compounds, to problems presently unrealized that will yield the largest impact on the scientific community. Though the intention of this research has always been to apply the principles of supramolecular chemistry to a couple of important problems of biochemical significance, the ultimate outcome of the work presented here will rest with those who apply the findings described to the problems of the future.
CHAPTER 10

MATERIALS, METHODS, AND EXPERIMENTAL PROCEDURES

10.1 Biophysical experiments

10.1.1 Instrumentation

All fluorescence spectroscopy was performed on either a Perkin Elmer LS50B or Jobin-Yvon FluoroMax-3 fluorimeter with FT WinLab software using standard 1x1x3 cm quartz cuvettes. UV-vis spectroscopy was performed with a PerkinElmer Lambda 2 spectrometer using standard 1x1x3 cm quartz cuvettes with a working volume of 1 mL. All spectrometers were connected to external water bath cooling units during experiments. Dynamic light scattering experiments were performed with a Coulter N4Plus instrument using a 1x1x3 cm quartz cuvette. DLS experiments were performed at room temperature.

10.1.2 Preparation of Phospholipid Vesicles

Experiments in which phospholipid vesicles were used (ie. fluorescence titration studies) were all conducted using vesicles prepared by the same procedure. All lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and stored as 10 mg/mL stock solutions in CHCl₃ at −20 °C. Lipids were added in the appropriate ratios to a 10 mL round bottom flask and solvent removed by rotary evaporation. Residual solvent was
removed under oil pump vacuum for \( \geq \) 1 hour. Dried lipid was then rehydrated in TES buffer (5 mM TES, 145 mM NaCl, pH 7.4, unless stated otherwise). A glass pyrex ring was added to the flasks to ensure complete removal of lipid from the flask walls, and then flasks were vortexed vigorously, followed by sonication when required to achieve complete suspension of the phospholipid. The resulting lipid dispersion was then extruded approximately 30 times through a 19 mm diameter polycarbonate membrane with 200 nm pore diameter (unless indicated otherwise). All vesicles were prepared at room temperature and used the same day.

10.1.3 Phospholipid Vesicle Binding Titration Experiments Using NBD-Labeled Compounds

Binding isotherms for all NBD-labeled Zn\(^{2+}\)-DPA compounds were determined according to the same procedure. A 3.0 mL volume of a 1.0 \( \mu \)M solution of each NBD-labeled compound was prepared in an aqueous buffer containing 5 mM TES and 145 mM NaCl at pH 7.4 in a quartz cuvette. Aliquots of the appropriate 10 mM vesicle suspension were added to the cuvette with vigorous stirring. NBD fluorescence was measured after each addition using excitation and emission wavelengths of 470 and 530 nm, respectively. A 515 nm cutoff filter was used in all NBD acquisitions. The resulting binding isotherms were then fit to a binding model describing a 1:1 association using the nonlinear regression function in Origin 6.0.
10.1.4 Carboxyfluorescein Leakage From Phospholipid Vesicles to DetermineMembrane Disruption

Vesicles of the appropriate composition were prepared as described, with the following exceptions. Lipids were rehydrated after drying using 5 mM TES buffer containing 50 mM (5-6)-carboxyfluorescein (155 mM overall). Following extrusion, vesicles were separated from excess carboxyfluorescein using a Sephadex G-75 column. Fluorimeter excitation and emission wavelengths were set to 495 and 520 nm, respectively, with an open filter. All measurements were performed at 25 °C without degassing of samples. The fluorescence intensity of a 3 mL sample of vesicles (50 μM total phospholipids) in 5 mM TES, 145 mM NaCl, pH 7.4 was monitored for 400 s. At time 100 s, the compound to be tested was added to the cuvette at the appropriate concentration, followed by addition of Triton X-100 detergent (0.5%) at time 300 s.

10.1.4 NBD Quenching by Sodium Dithionite to Determine Membrane Permeability

Vesicles of the appropriate composition were prepared as described earlier. At time 0 s, an appropriate volume of NBD-labeled compound was injected into 3 mL of a 30 μM solution of vesicles. At time 50 s, sodium dithionite was added (60 mM final concentration) to quench all external NBD-labeled compound. At time 150 s, an injection of Triton X-100 detergent (0.5%) was made to disrupt all vesicles and allow all internalized NBD-labeled material to be quenched. Fluorescence intensities were collected every second for the entire 200 s interval.
10.1.5 Dynamic Light Scattering

Dynamic light scattering experiments were performed in an aqueous buffer containing 5 mM TES and 145 mM NaCl at pH 7.4. A 3.0 mL volume of buffer was filtered through a 200 nm pore diameter filter and placed in a 1x1x3 cm quartz cuvette. To this solution was added 5 µL of a phospholipid vesicle suspension containing 10 mM total lipid. Aliquots of a 1 mM aqueous solution of the Zn$^{2+}$-DPA compound to be tested were added to the vesicle suspension in the cuvette and the average particle diameter recorded after each addition.

10.2 Indicator Displacement Assays for PS-Rich Membranes

10.2.1 General

Indicators 17 and 19 were purchased from Aldrich and used without further purification. Coumarin methylsulfonate 18 was prepared by Dr. Hua Jiang. All experiments were performed at 25 °C in 1 x 1 x 5 cm quartz cuvettes. All nonlinear curve fitting for determination of association constants was performed using Origin 6.0, and all determination of association constants for phospholipid vesicle binding to receptors was done using Microsoft Excel.

10.2.2 Determination of Association Constants for Binding of 17 by the Zn$^{2+}$-DPA Compounds

A 1.0 mL aqueous solution of 17 (50 µM final concentration) was prepared in a buffer of 5 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 145 mM NaCl, pH 7.4. Aliquots of an aqueous 10 mM Zn$^{2+}$-DPA receptor solution in the
same buffer containing 50 µM 17 were titrated into the indicator solution and dispersed thoroughly by repeated pipet mixing. Aliquots were added until the Zn\(^{2+}\)-DPA receptor concentration reached 400 µM. After each addition, the UV-Visible absorbance spectrum of the solution was acquired. Plots of absorbance values (600 nm) as a function of Zn\(^{2+}\)-DPA receptor concentration were generated for the addition of 13-16 and fit to a 1:1 binding model to determine the receptor-indicator association constant for receptors 15 and 16. Binding of 13 and 14 to 17 was found to be too tight for accurate determination of an association constant.

10.2.3 Determination of Association Constants for Binding of 18 by the Zn\(^{2+}\)-DPA Compounds

A 3.0 mL aqueous solution of 18 (10 µM final concentration) was prepared in a buffer of 5 mM TES, 145 mM NaCl, pH 7.4. While stirring, aliquots of an aqueous 10 mM Zn\(^{2+}\)-DPA receptor solution in the same buffer were titrated into the indicator solution until the final receptor concentration reached 100 µM. After approximately 30 s for solution equilibration, the fluorescence emission intensity of 18 was measured (\(\lambda_{\text{ex.}} = 347\) nm, \(\lambda_{\text{emiss.}} = 480\) nm) after each addition. Plots of fluorescence intensity as a function of Zn\(^{2+}\)-DPA receptor concentration were generated for the addition of 13-16 and fit to a 1:1 binding model to determine the receptor-indicator association constant for each receptor.
10.2.4 Procedure for Performing Displacement Assays

*UV-Vis assay based on indicator 17:*

To a 3.0 mL volume of a 1:1 mixture of Zn$^{2+}$-DPA receptor (13-16) and indicator 17 (50 µM each) in an aqueous buffer of 5 mm TES, 145 mM NaCl, pH 7.4, were added aliquots of a 10 mM suspension of 200 nm POPC vesicles containing between 0 and 50% POPS until the total phospholipid concentration reached 400 µM. The vesicle suspension contained 50 µM 5 in order to offset the dilution effect realized during titration. After repeated pipet mixing, the absorbance spectrum of the solution was acquired.

*Fluorescence assay based on indicator 18:*

To a 3.0 mL volume of a 1:1 mixture of Zn$^{2+}$-DPA receptor (13-16) and indicator 18 (10 µM each) in an aqueous buffer of 5 mM TES, 145 mM NaCl, pH 7.4, were added aliquots of a 10 mM suspension of 200 nm POPC vesicles containing between 0 and 50% POPS until the total phospholipid concentration reached 100 µM. After approximately 30 s for solution equilibration, the fluorescence emission intensity of 18 was measured ($\lambda_{\text{ex.}} = 347$ nm, $\lambda_{\text{emiss.}} = 480$ nm) after each addition. The intensity values as a function of total phospholipid concentration were then used to determine a receptor-phospholipid membrane association constant by methods previously described.

10.3 CFTR Aggregate Separation by SDS-PAGE Electrophoresis

The CFTR transmembrane four peptide was dissolved in a buffer of 5 mM TES, 25 mM NaCl, pH 9.15 at a concentration of 2 mg/mL, and left exposed to the atmosphere at room temperature for approximately 14 days to allow for oxidation of the C225 residues and formation of the requisite disulfide bond. Shorter periods resulted in
substantially less oxidation and almost negligible quantities of higher order oligomerization.

Samples for SDS-PAGE were prepared by adding 2.0 µL of the fully oxidized peptide in aqueous solution (2 mg/mL) to the appropriate volume of a solution of the molecule to be tested. Sample buffer (Invitrogen, 2.0 µL) was added, along with a sufficient volume of distilled water to bring the total sample volume to 10.0 µL. Samples were then incubated at room temperature for 30 minutes. All gels used were commercially available precast 4-12% Bis-Tris Polyacrylamide (Invitrogen). Approximately 2 µg of peptide, the entire 10 µL sample volume, was loaded onto the gel following incubation and run at room temperature in 1X Running Buffer (Invitrogen) according to the manufacturer’s recommendations. Gels were stained for approximately 45 minutes with Coomassie Blue and visualized by standard techniques.

10.4 DAGK Inhibition Assay

The coupled assay for inhibition of DAGK was performed in an aqueous buffer containing 60 mM PIPES at pH 6.85. To a 10 mL round bottom flask was added the necessary volume of cardiolipin and dioleoylglycerol (Avanti Polar Lipids) as CHCl₃ stock solutions. The solvent was removed by rotary evaporation and residual solvent removed under vacuum for at least one hour. Lipids were then rehydrated with the buffer indicated above and removed from the flask wall by vortex and sonication to produce a cloudy suspension.

Each assay mixture contained the following components in the amounts or concentrations indicated: cardiolipin (1.6 mg), dioleoylglycerol (0.4 mg), β-octyl-D-
glucopyranoside (12 mg), phosphoenolpyruvate (2 mM), ATP-magnesium salt (5 mM), MgCl₂ (20 mM), pyruvate kinase (18 units), lactate dehydrogenase (22 units), and any inhibitor to be tested at the appropriate concentration. The volume of the assay mixture was adjusted to 1.0 ml with the assay buffer. This solution was then used to blank the UV spectrophotometer for absorbance readings at 340 nm. NADH (0.3 mM) was then added to the solution and the UV absorbance at 340 nm monitored until the value of A₃₄₀ remained approximately constant, at which point DAGK (10 µg) was added to the solution. The reaction was allowed to proceed until the value of A₃₄₀ decreased to approximately 0.00. The value for t₁/₂ was determined as the time required for the value of A₃₄₀ to decrease by a factor of two from the initial value determined at the time of addition of DAGK.

10.5 Cell Staining and Fluorescence Microscopy

Annexin V and 7AAD were obtained from BD Biosciences. Quantum dot-streptavidin conjugates were from Quantum Dot Corporation (Hayward, CA). Marina Blue streptavidin conjugate was from Molecular Probes (Eugene, OR). Jurkat cells were grown to a density of approximately 1.0 x 10⁶ per mL in RPMI 1640, 10% FCS at 37 °C, 5% CO₂. A 10 mL volume of cells was treated with camptothecin (10 µM final concentration) in growth media for 3.5 h at 37 °C, 5% CO₂. Cells were spun down and resuspended in 1X annexin binding buffer (10 mM HEPES - sodium salt, 2.5 mM CaCl₂, 140 mM NaCl, pH 7.4) for experiments in which annexin V was used, or in a buffer of 5 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 145 mM NaCl, pH 7.4 for experiments in which annexin V was not used. Aliquots (0.5 mL) of the treated
cells, along with controls, were then treated with the indicated staining reagents at the indicated concentrations. Annexin V-FITC was used according to the manufacturer’s protocol (BD Biosciences). All reagents were added simultaneously. The cell suspensions were mixed thoroughly by repeated inversion and then incubated 15 minutes at 37 °C, except where temperature effects were being evaluated. Cells were then centrifuged, resuspended and washed twice in 5 mM TES, 145 mM NaCl, pH 7.4 buffer. At this point, 250 µL of the suspension was transferred to a 16 well chamber slide for microscopy. Fluorescence microscopy was performed immediately following cell staining on an Axiovert S100 TV microscope (Carl Zeiss) equipped with filter sets DAPI/Hoechst/AMCA, FITC/RSGFP/Bodipy/Fluo3/DiO, Cy3 (Chroma). Pictures were taken using a black and white digital camera and colored afterwards using Photoshop 6.0 software package (Adobe).

10.6 Flow Cytometry

Jurkat cells were cultured according to the same procedures described for fluorescence microscopy. A 10.0 mL volume of cells was treated with camptothecin (10 µM final concentration) in growth media for 16.5 h at 37 °C, 5% CO₂. Cells were spun down and resuspended in 1X annexin binding buffer (10 mM HEPES - sodium salt, 25 mM CaCl₂, 140 mM NaCl, pH 7.4) for experiments in which annexin V was used, or in a buffer of 5 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 145 mM NaCl, pH 7.4 for experiments in which annexin V was not used. Cell aliquots (1.0 mL) were stained with 7AAD (500 ng/mL) and either PSS-480 (5 µM) or annexin V-FITC (5 µL/mL – BD Biosciences commercial solution). All reagents were added
simultaneously. The cell suspensions were mixed thoroughly by repeated inversion and then incubated 15 minutes at 37 °C, except where temperature effects were being evaluated. Cells were then centrifuged, resuspended and washed twice in 5mM TES, 145 mM NaCl, pH 7.4 buffer. Flow cytometry was performed immediately after staining on an Epics XL flow cytometer (Coulter, Miami, FL) with an argon laser. FITC was analyzed using a 520 nm bandpass filter, and 7AAD was analyzed using a 580 nm bandpass filter. Software color compensation was used and data analysis was performed using Multiplus AV Software (Phoenix Flow Systems).

10.7 Synthesis
10.7.1 General

Unless otherwise stated, all reagents were purchased from Aldrich Chemical Company and used without further purification. All NMR chemical shift values are based on spectra referenced to solvent residual peaks. NMR spectra were recorded on a Varian spectrometer.

10.7.2 Synthesis of Thyminedodecyl Bromide (22a)

The thyminedodecyl bromide 22a shown in Scheme 1 was a key intermediate for synthesis of nearly all the thyminedodecyl analogs. Thymine (1.00 g, 7.9 mmol) was added to a three-necked round bottom flask equipped with a nitrogen inlet and stir bar. To this flask was added 1,1,1,3,3,3-hexamethyldisilazane (5.16 mL, 24.7 mmol) and trimethysilylchloride (0.516 mL, 4.1 mmol). The mixture was heated at reflux for 24 h, during which a white film of ammonium chloride formed in the neck of the flask and the
reflux condenser. Residual HMDS was removed by rotary evaporation to yield a pale yellow semisolid, which was dissolved in 3.3 mL DMF. 1,12-dibromododecane (8.26 g, 30.4 mmol) was then added and the mixture heated to 130 °C for 24 h under nitrogen. A 50 mL portion of icewater was then added and extracted with two 50 mL portions of methylene chloride. The combined methylene chloride layers were concentrated by rotary evaporation to yield a viscous dark red oil, which was then taken up in 50 mL chloroform. Pentane was slowly added to precipitate 22a and unreacted 1,12-dibromododecane. After filtration the resulting solid was applied to a silica gel column and eluted with gradient elution using chloroform containing 0-2% methanol as the eluant. The product, 22a, (678 mg, 1.8 mmol) was obtained as an off-white solid in 23% overall yield. \( ^1 \)H NMR (300 MHz, CDCl\(_3\)): \( \delta \) 9.63 (s, 1H), 6.96 (s, 1H), 3.66 (t, \( J=7.5 \) Hz, 2H), 3.38 (t, \( J=6.9 \) Hz, 2H), 1.89 (s, 3H), 1.82 (quint., \( J=7.2 \) Hz, 2H), 1.39 (m, 2H), 1.64 (m, 2H), 1.24 (m, 14H). \( ^{13} \)C NMR (75 MHz, CDCl\(_3\)): \( \delta \) 164.6, 151.1, 140.4, 110.5, 48.5, 34.1, 32.8, 29.4, 29.3, 29.1, 29.0, 28.7, 28.1, 26.4.

10.7.3 Synthesis of Thyminedodecyltrimethylammonium Bromide (22)

22a (250 mg, 0.67 mmol) was dissolved in 10 mL DMF, capped with a rubber septum, and stirred in an ice bath for 30 minutes. A gas inlet needle was inserted through the septum and trimethylamine slowly allowed to bubble through the solution for approximately one minute. The reaction was then allowed to stir for approximately twenty minutes, after which time trimethylamine was again bubbled through the solution for approximately one minute. This was repeated approximately eight times and the reaction was then allowed to stir for two hours, eventually coming to room temperature.
The rubber septum was removed and the solution stirred at room temperature for 30 minutes. Diethyl ether was then slowly added to the solution to precipitate 22 (100 mg, 0.23 mmol) as a white solid in 35% yield. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.50 (s, 1H), 3.74 (t, $J$=6.9 Hz, 2H), 3.29 (m, 2H), 3.09 (s, 9H), 1.86 (s, 3H), 1.76 (m, 2H), 1.65 (m, 2H), 1.24 (m, 16H).

$^{13}$C NMR (75 MHz, D$_2$O): $\delta$ 166.7, 152.0, 143.2, 110.3, 66.7, 52.8, 48.5, 28.8, 28.7, 28.5, 28.4, 28.2, 25.6, 25.5, 22.3, 11.4.

10.7.4 Synthesis of $N$-methyl-thyminedodecyl Bromide (23a)

22a (253 mg, 0.68 mmol) was dissolved in 3 mL DMF. K$_2$CO$_3$ (325 mg, 2.35 mmol) was added and the mixture stirred for 30 minutes at room temperature.

Methyliodide (500 µL, 8.05 mmol) was then added and the reaction mixture stirred at room temperature for 3.5 d. Undissolved K$_2$CO$_3$ was then removed by filtration and the solvent removed by rotary evaporation. The resulting yellow oil was taken up in 10 mL CHCl$_3$ and the remaining K$_2$CO$_3$ was removed by filtration. Removal of the CHCl$_3$ by rotary evaporation gave 23a as a yellow oil in near quantitative yield (>99%, 263 mg, 0.68 mmol). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 6.97 (s, 1H), 3.70 (t, $J$=7.5 Hz, 2H), 3.56 (s, 3H), 3.19 (t, $J$=7.2 Hz, 2H), 1.94 (s, 3H), 1.82 (quint., $J$=6.6 Hz, 2H), 1.67 (m, 2H), 1.59 (m 2H), 1.26 (m, 14H). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 164.4, 151.5, 138.3, 109.4, 49.6, 33.5, 30.4, 29.4, 29.3, 29.2, 29.1, 28.5, 27.9, 26.5, 13.1, 7.3.
Scheme 1
10.7.4 Synthesis of $N$-methyl-thyminedodecyltrimethylammonium Bromide (23)

23a (100 mg, 0.26 mmol) was dissolved in 10 mL DMF, capped with a rubber septum, and stirred in an ice bath for 30 minutes. A gas inlet needle was inserted through the septum and trimethylamine slowly allowed to bubble through the solution for approximately one minute. The reaction was then allowed to stir for approximately twenty minutes, after which time trimethylamine was again bubbled through the solution for approximately one minute. This was repeated approximately eight times and the reaction was then allowed to stir for two hours, eventually coming to room temperature. The rubber septum was removed and the solution stirred at room temperature for 30 minutes. Diethyl ether was then slowly added to the solution to precipitate 23 as a yellow solid suspended in solution. Cooling the solution at 0 °C for six hours followed by filtration afforded 23 as a yellow solid (42 mg, 0.09 mmol) in 35% yield. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.65 (s, 1H), 3.78 (t, $J$=6.6 Hz, 2H), 3.40 (m, 2H), 3.27 (s, 3H), 3.21 (s, 9H), 1.90 (s, 3H), 1.80 (m, 2H), 1.64 (m, 2H), 1.23 (m, 16H). $^{13}$C NMR (75 MHz, D$_2$O): $\delta$ 165.5, 151.9, 140.9, 109.0, 66.6, 53.1, 49.5, 29.1, 29.0, 28.8, 28.7, 28.5, 28.1, 26.0, 25.8, 22.6, 12.4.

![Scheme 2](image_url)

**Scheme 2**
10.7.6 Synthesis of Thymine-12-dodecylsulfite (24)

The primary bromide 22a (259 mg, 0.69 mmol) and Na$_2$SO$_3$ (102 mg, 0.81 mmol) were taken up in 25 mL distilled H$_2$O and heated to 140 °C for 24 h. After cooling to room temperature, the solution was filtered to remove insoluble material, and then the water removed by rotary evaporation to give a white solid. The white solid was taken up in 30 mL methanol and filtered to remove the insoluble inorganic salts, then the methanol was removed by rotary evaporation to yield 24 (96 mg, 0.26 mmol) as a white powder in 37% overall yield. $^1$H NMR (300 MHz, CDCl$_3$): δ 1.23 (br s, 16 H), 1.54 (m, 4 H), 1.74 (s, 3 H), 2.38 (m, 2 H), 3.50 (t, $J$=7.2 Hz, 2 H), 7.54 (s, 1 H), 11.18 (br s, 1 H). $^{13}$C NMR (75 MHz, D$_2$O): δ 11.9, 25.0, 25.8, 28.4, 28.6, 28.9, 29.0, 47.1, 51.1, 108.3, 141.6, 150.9, 164.3.

10.7.7 Synthesis of 1-(12-hydroxydodecyl)thymine (25)

Thymine (164 mg, 1.30 mmol) was added to a three-necked round bottom flask equipped with a nitrogen inlet and stir bar. To this flask was added 1,1,1,3,3,3-hexamethyldisilazane (5.0 mL, 23.98 mmol) and trimethylsilylchloride (0.450 mL, 3.6 mmol). The mixture was heated at reflux for 24 h, during which a white film of ammonium chloride formed in the neck of the flask and the reflux condenser. Residual HMDS was removed by rotary evaporation to yield a pale yellow semisolid, which was dissolved in 10 mL DMF. 12-bromo-1-dodecanol (340 mg, 1.3 mmol) was then added and the mixture heated to 130 °C for 24 h under nitrogen. After cooling to room temperature, solvent was removed by rotary evaporation to give a red solid, which was taken up in CHCl$_3$ and filtered to remove insoluble material. The remaining CHCl$_3$
solution was concentrated and purified over a silica column using CHCl$_3$ containing 0 to 3% methanol. The content of the first substantial band collected was combined and purified over another silica column using a mobile phase of 100% CHCl$_3$. Compound 25 was collected as a white solid (16.8 mg) in 4.2% overall yield. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 1.24 (br s, 12H), 1.53 (m, 2H), 1.63 (quint. $J = 7.2$ Hz, 2H), 2.05 (s, 3H), 3.59 (t, $J = 6.3$ Hz, 2H), 4.13 (t, $J = 6.6$ Hz, 2H), 8.03 (s, 1H).
REFERENCES


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