INTERACTION OF CELL-PENETRATING MACROMOLECULES WITH MODEL MEMBRANES

Venkataswarup Tiriveedhi
University of Southern Mississippi

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WITH MODEL MEMBRANES

by

Venkataswarup Tiriveedhi

A Dissertation
Submitted to the Graduate Studies Office
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Approved:

December 2007
The University of Southern Mississippi

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ABSTRACT

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The general class of cationic cell-penetrating macromolecules (CPMs) is defined by their ability to translocate into the living cells in a rapid manner by as yet unknown mechanism. While our long-term goal is the elucidation of the mechanism, the present work focused on the first phase of the translocation, which is binding of CPMs to the model cell membrane. We used fluorescence spectroscopy to study the interaction between CPMs and the lipid bilayers and monolayers labeled with various fluorescence probes. The two CPMs we studied are protein transduction domain (PTD) of HIV-1 Tat protein (TAT-PTD; residues 47-60 of Tat) and lower generations polyamidoamine (PAMAM) dendrimers G1 and G4 (generation 1, 4) labeled with various fluorophores. Kinetic analysis of the interaction between TAT-PTD and lipid bilayer, showed two apparent dissociation constants. While the value of one dissociation constant (Kd1), was found to be independent of the negative charge density, the value of the second dissociation constant (Kd2), decreased linearly with increasing negative charge density from zero to 25 mol%, suggesting a non-electrostatic and electrostatic nature of this interaction, respectively. However, salt studies with compact dendrimers showed the interactions to be completely electrostatic. Fluorescence resonance energy transfer (FRET), quenching, temperature-dependence and pyrene-labeled SUV experiments suggested that TAT-PTD is always on the outer surface of the lipid bilayer. Similar
studies with dendrimers suggested a dendrimer-induced aggregation of lipid vesicles.
Both TAT-PTD and PAMAM dendrimers preferentially bound to the membrane in the liquid state; only in that state could they gather multiple negatively charged lipid molecules, causing CPM-induced phase separation of lipids into distinct microdomains. Experiments with dendrimers showed a significant increase in surface pressure when initial pressure was below 20 mN/m, and no significant increase at physiologic surface pressure (> 29 mN/m). Hence, dendrimers are able to insert into monolayers at low pressure, but not at physiologic one. We conclude that the two studied CPM interact predominantly with the polar surface of the model lipid membrane, without significant disruption of membrane integrity. The electrostatics and membrane fluidity are the most important physico-chemical parameters that govern the membrane binding of these CPM.
ACKNOWLEDGEMENT

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I dedicate this work to my parents Venkataswamy Tiriveedhi and Saraswathi Devi Tiriveedhi for all the love and sacrifices they made for the success of their children.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>A°</td>
<td>Angstrom ($10^{-10}$ meters)</td>
</tr>
<tr>
<td>A$_{280}$</td>
<td>Absorbance at 280 nm</td>
</tr>
<tr>
<td>A$_{\text{max}}$</td>
<td>Absorbance at maximum wavelength</td>
</tr>
<tr>
<td>arb. units</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>CoBr$_2$</td>
<td>Cobalt bromide</td>
</tr>
<tr>
<td>CPP(s)</td>
<td>Cell-Penetrating peptides</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton (g/mol)</td>
</tr>
<tr>
<td>DPH</td>
<td>1,6-diphenyl-1,3,5-hexatriene</td>
</tr>
<tr>
<td>DiSC$_3$(5)</td>
<td>3,3'-dipropylthiadicarbocyanine iodide</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DMPG</td>
<td>1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]</td>
</tr>
<tr>
<td>DPPS</td>
<td>dipalmitoyl phosphatidylserine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo nucleic acid</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>$F$</td>
<td>Faraday constant</td>
</tr>
<tr>
<td>F360</td>
<td>Fluorescence intensity at 360 nm</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo (10^3) Daltons</td>
</tr>
<tr>
<td>Ksv</td>
<td>Stern-Volmer quenching constant</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicles</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>R</td>
<td>gas constant</td>
</tr>
<tr>
<td>NATA</td>
<td>N-acetyl tryptophanamide;</td>
</tr>
<tr>
<td>pyrene-PG</td>
<td>1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol</td>
</tr>
<tr>
<td>TMA-DPH</td>
<td>1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>T</td>
<td>absolute temperature</td>
</tr>
<tr>
<td>Tat</td>
<td>Trans-acting activator of transcription</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>PC</td>
<td>L-α-phosphatidylcholine</td>
</tr>
<tr>
<td>PG</td>
<td>L-α-phosphatidylglycerol</td>
</tr>
<tr>
<td>PTD</td>
<td>Protein-transduction domain</td>
</tr>
<tr>
<td>pyrene-PG</td>
<td>1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol</td>
</tr>
<tr>
<td>rel. units</td>
<td>Relative units</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>Z</td>
<td>charge</td>
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$\varepsilon$  molar extinction coefficient

$\Delta \psi$ membrane potential
CHAPTER 1

General Introduction

Over the last several years, certain natural or natural-derived peptides and proteins and synthetic polymers have been shown to penetrate the cell membrane, enter the cell, and reach the nucleus, while retaining the cell's normal biological activity, by a process called transduction (1-6). This array of complex macromolecules are designated under a common class called cell-penetrating macromolecules (CPMs). In general, CPMs are highly charged macromolecules that traverse the biological membranes within minutes. These macromolecules have been shown to be both naturally occurring (7) and synthetic (8). They have a promising future as potential drug delivery vehicles (9, 10) and cell transfection agents (11, 12). This offers a lot of hope, in that the CPMs will enhance efficiency in delivery issues, such as targeting, stability and bioavailability.

The current understanding of the mechanisms of the interaction of CPMs with bilayer lipid membranes is quite meager. As it stands at present, the field is still in its infancy, and the current literature offers very little concrete and rigorous knowledge on how the delivery is occurring at the molecular level. The major hindrance in the above quest may be the complexity of the biomembrane matrix and the diversity in chemical nature of CPMs, which makes it difficult to interpret results.
1.1 Classification of CPMs

There is no consensus for a unified classification of CPMs in the literature, but for illustration purposes we use the following method of classification in our discussion (Figure 1.1). The two main groups of CPMs are peptides or protein derivatives (CPPs) and non-peptide chemically synthesized polymers. The peptide group can be further divided into derivatives of naturally occurring proteins and synthetic peptides (13). Some naturally occurring CPMs are represented by short amino-acid sequences found within proteins from various sources: HIV-1 TAT (trans activator of transcription) protein, Ant P penetratin of Drosophila antennapedia homeoprotein, vp22 from herpes virus, or the flock house virus coat protein (1, 14). Synthetic peptide CPMs include transportan, a peptide adapted from the neuropeptide galanin, or SynB peptide derived from the antimicrobial protein protegrin (7, 15, 16). Homopolymer sequences of polylysine, polyarginine, and polyornithine are also included in this group (17-19). The non-peptide, synthetic-polymer -based CPMs can be classified into linear polymers and globular dendrimers (8, 20).

FIGURE 1.1: Classification of CPMs.
1.2 Cell-Penetrating Peptides (CPPs)

1.2.1 CPP overview

A common classification and terminology of the membrane-crossing peptides is yet to be developed (21). However, the commonly used name “cell-penetrating peptide” (CPP) was introduced in a review article (7) and the first book on this particular topic (22). Based on the recently suggested classifications, CPPs can be placed in three classes: protein derived CPP, model peptides, and designed CPPs (21). Protein derived CPPs are minimal effective partial sequences of parent proteins also called protein transduction domains or membrane translocation sequences. Model CPPs are designed sequences with the purpose of producing well-defined structure of similar polarity mimicking that of known CPPs. Chimeric peptides composed of hydrophobic and hydrophilic domains of different origin are classified as designed CPPs. Some naturally occurring CPPs are derived from HIV-1 TAT (trans activator of transcription) protein, Ant P penetratin of Drosophila antennapedia homeoprotein, vp22 from Herpes virus, or the flock house virus coat protein. Synthetic CPPs include transportan, a synthetic peptide adapted from the neuropeptide galanin, or SynB peptide derived from the antimicrobial protein protegrin (7, 15, 16). As can be noticed, CPPs from different classes do not share a common amino-acid sequence motif. An apparent common feature in all CPPs appears to be the positive charge. All CPPs known to date have a net positive charge at physiologic pH, with the contents of basic amino acids ranging from approximately 17%, in hCT(9-32) (23) to 100% in polyarginines (17, 24).
1.2.2 Reason for attention towards CPP molecules

Due to the diverse chemical nature of various CPPs, there is no reason to assume a unified mechanism of their action. The transport of CPPs across membrane has been studied using cultured cells, artificial lipid vesicles, tissues (in situ) and in whole animals (in vivo). CPPs were successfully internalized in primary cells such as those of rat brain and rat spinal cord (1), calf aorta (25), porcine and human umbilical vein endothelium (26), and in osteoclast cultures (27). No special cell cultivating procedures are needed for internalization studies with cell lines. Internalization in fixed cells is monitored directly using confocal microscopy and other imaging techniques (28, 29). It has been recently argued that even mild fixating agents like formaldehyde and paraformaldehyde can produce fixation artifacts (6). However, internalization of penetratin, Tat-PTD, and transportan was detected in both fixed and live cells from several cell lines (Bowes, Jurkat, Hela, Caco-2) (6, 15, 30-33).

1.2.3 The CPP controversy

In spite of the often astonishing claims in the literature, the exact biochemical and biophysical mechanism involved in the internalization of CPPs is still a matter of great debate. Several studies were conducted under conditions that would normally prevent active transport of CPPs and their translocation by endocytosis. Efficient translocation was observed both at low temperatures (0 to +4°C) and in the presence of the inhibitors of classical endocytosis (1, 14, 15, 26, 31, 34-37). Internalization is reported to occur with D as well as with L stereoisomers of the peptides (38), which is used to support the notion that no receptor is required for the process. These studies have suggested the rapid
internalization of CPPs into the cell in an apparently energy-independent manner. However, more recent studies have shown that the role of endocytosis may not be negligible (39, 40). Studies have shown that internalization of penetratin and protegrin-1 derived SyrB peptides into the live cells is related to endocytotic processes (41) and that Tat-derived CPPs do not internalize into the cells (6) or liposomes (42) at low temperature. Recently it was suggested that Tat-derived CPPs require macro-pinocytosis, mediated by lipid rafts, for their mechanism of action (43). An ‘inverted micelle’ mechanism of action was suggested for Tat-derived CPPs and penetratin (Figure 1.2), wherein the positively charged peptides interact with negatively charged phospholipids to convert part of the membrane structure into an inverted micelle (1, 40). Other suggested mechanisms like ‘carpet model’ (for pVEC and transportan analogues), and ‘α-helix’ formation (for MAP and partly transportan) might implicate a possible pore formation (44-47).

FIGURE 1.2: Proposed mechanism of internalization of CPPs (38, 48).
1.3 TAT Protein Transduction Domain (TAT-PTD)

1.3.1 TAT-PTD overview

Tat protein is one of the fifteen distinct proteins in human immunodeficiency virus type 1 (HIV-1), a protein of 101 or 86 amino acids, depending on C-terminal cleavage observed in some laboratory HIV strains (49). TAT protein binds to its receptor on the cell surface, translocates into the cell, and concentrates close to the nuclear membrane (49). The main function of this protein is to potentiate the viral DNA insert into mRNA carried out by the host cell RNA polymerase (50, 51). Without TAT, the host cell polymerase complex stops elongation of the viral genome after approximately 100 nucleotides (52).

From the biophysical-biochemical point of view Tat protein is divided into six regions (3)—namely the acidic (residues 2-11) and cysteine-rich regions (residues 22-37), the hydrophobic core (residues 38-48), the basic (residues 49-57) and the glutamine-rich regions (residues 58-72), and the RGD motif (residues 72-86) (53-55). The basic region of the Tat binds to the negatively charged mRNA, and this binding becomes specific for Tat activating region (TAR) when the hydrophobic core is flanking the basic region. The cysteine-rich region of Tat, in turn, is important for the activation of a complex containing kinases, an elongation factor, and a polymerase, an activation, which occurs even without other regions of Tat. When handling the larger amounts of Tat it should be realized that Tat (residues 31-61) exhibits neurotoxicity in a dose dependent
manner (55, 56). Small fragments of Tat, comprising the basic region have been found to pass through biological membranes in vivo very efficiently by still unknown mechanism.

The initial studies in the late eighties have shown that the recombinant HIV-1 Tat protein was taken up by cells and promoted nuclear transactivation of a reporter gene under the control of HIV-1 long terminal repeat (LTR) (3). A few years later an identical property was described for a sixty amino-acid sequence derived from the Drosophila Antennapedia homeodomain (5). A preliminary structure evaluation has showed a hexadecamer peptide sequence responsible for this translocating activity (1). When the enzyme β-galactosidase was attached to this sequence of Tat protein, the whole covalent complex was taken up by the cells (57). Similar control studies with fluorochrome (14) and other peptides did not show any (14, 33, 38). However, these studies were carried out using fluorescence microscopy, with formaldehyde as the fixating agent (38). This led to the questioning the reality of this unusual translocation behavior of Tat-derived and other similar protein and peptide derivatives, as against being an experimental artifact. The translocation of other CPPs at 4°C and the indifference to D and L stereochemistry of the peptides warrant a lot of attention and closer look into this process.

1.3.2 Possible mechanisms of internalization

It is still not clear how CPPs in general, and TAT-PTD in particular, enter the cell. Proteins, peptides, ions and small molecules can enter cells through a variety of mechanisms. Specialized pumps and channels on the cell surface are known to assist the transport of some of these molecules. Macromolecules in general are transported into the
cell by endocytosis. Endocytosis occurs by multiple mechanisms that are broadly classified into two categories: phagocytosis and pinocytosis. Phagocytosis usually refers to uptake of large particles and is restricted to some specialized cells like macrophages and neutrophils. Pinocytosis occurs in most of the cells and refers to uptakes of fluids, ions and some small particles. Four common mechanisms have been identified that contribute to pinocytosis: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-, caveolae-, and dynamin-independent endocytosis. However, the differentiation between the different types of pinocytosis is subtle and beyond the scope of the present discussion (58).

Interestingly, several apparently conflicting results were published in recent literature. Some researchers hypothesized that clathrin-dependent endocytosis plays a role in uptake of TAT-PTD (6), whereas some other studies using C105Y (a CPP derived from α1-antitrypsin) showed that incubation with chlorpromazine, a known inhibitor of clathrin-dependent endocytosis, did not inhibit the uptake (59). Furthermore, these contradictory results also put the originally claimed notion—that the translocation of CPP is energy independent—into question, the major reason being that clathrin-dependent endocytosis is energy-dependent. Another hypothesis—that endocytosis mediated by lipid rafts is a prerequisite for internalization—seems controversial as well. Broadly, lipid rafts are sub-divided into two distinct categories: caveolae or caveolin-containing lipid rafts, and clathrin- and caveolin-independent lipid rafts. Internalization of C105Y was shown in HuH7 cell lines, which express very low levels of caveolin-1 and 2 (Cav-1 and Cav-2), and also in caveolin knock-out mutant cell lines (59). However, the very same group (59) showed that internalization of C105Y is inhibited when cells were incubated...
with methyl-β-cyclodextrin, which is a known inhibitor of all lipid raft dependent pathways by removing cholesterol. This study shows that lipid rafts play a key role in internalization of CPPs. However, another research group showed that methyl-β-cyclodextrin did not inhibit the internalization of TAT-PTD (6). These apparently contradictory results may be due to different mechanisms of internalization utilized by various CPPs, or might be suggestive for further research towards a consensus hypothesis, which has completely skipped the attention of various researchers, or both.

Most of the above said studies have used fluorescence or other microscopy technique, which involves cell fixation. The validity of some of the important conclusions drawn from the data has been questioned by several authors (6, 60, 61). It has been shown that the energy and temperature independence of cellular uptake of TAT-PTD, as well as its fast nuclear accumulation, might have resulted from experimental artifacts due to cell fixation and incomplete removal of cell-bound peptide (6, 61). Literature evidence suggesting that internalization of CPPs is temperature- and energy-independent and the lack of specific receptors lead to one possible hypothesis, namely, that these highly charged cationic and hydrophilic CPPs, directly translocate through the hydrophobic lipid membrane. However, if true, as noted by Richard et al. (6), this hypothesis would warrant 'a radical revision' of current understanding on the properties of lipid bilayers because: the passive transport of a highly charged large peptides through the hydrophobic core of the lipid bilayer is energetically extremely costly. Indeed, it was shown that TAT-PTD mediated only cell binding, and not the complete translocation, of the diphtheria toxin A-fragment (62).
Alternatively, theories have been proposed suggesting a complex formation between the cationic CPPs and anionic macromolecules as a way to neutralize effective charge on the peptide. Some researchers have postulated the posible role of glycosaminoglycans, like heparan sulfate, which are constituents of the extracellular matrix (3, 63, 64), or simply the anionic lipids of the cell membrane (38) as potential complex-forming agents. However, neither this complex formation hypothesis is not without controversy, as it could not be verified by some research groups (65).

### 1.3.3 Applications in intra-cellular drug delivery

The main applicative potential of CPPs is the possibility of attaching biologically active molecules and translocating them into the cell. The cargo molecule should be covalently attached to the CPP. If the cargo molecule is another peptide, it can be synthesized simultaneously with the synthetic CPP. Alternatively, reactive groups, like the thiol of cysteine, have been used for covalent bond formation (66). Strong non-covalent bonds can also be utilized by using a avidin/biotin-CPP construct. Despite questions about their mechanism, CPPs have a great potential in biotechnology applications. They are able to carry along wide variety of covalently attached molecules, such as enzymes of molecular weight up to 120 kDa, liposomes with the diameter of 200 nm, 40-nm magnetic nanoparticle beads and DNA phages (10, 67, 68). Thus, CPPs are capable of increasing or enabling uptake and possible nuclear targeting of biologically active large molecules. This makes them a desirable candidate for intracellular drug delivery and rapid nonviral gene transfer systems.

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1.4 Dendrimers

1.4.1 Introduction

Dendrimers, highly branched complex macromolecules, were discovered in early 1980’s, their name being derived from the word ‘dendron’, which is Greek for ‘tree’ (69, 70). Several names, like arborals or cascade molecules, were used initially, but ‘dendrimers’ is the present established term. Poly-amidoamine (PAMAM) dendrimers with amine termini, also called star-burst dendrimers, are the first synthesized dendrimers that extend up to 10 generations (71, 72), that is, they have up to 10 consecutively more and more branched layers, which surround the core moiety. Due to their unique water solubility, they have been of particular interest in the biomedical field for their potential use in the intracellular drug delivery (73-75). Similarly to CPPS, they have been shown to transfer DNA fragments (76-79) immunoglobulins (80), and anticancer drugs (81) across the cell membrane.

1.4.2 Synthesis

PAMAM dendrimers with ethylenediamine core can be prepared by either convergent or divergent methods, with an exponential increase in the number of the surface charged groups (82). Poly (amidoamine) (PAMAM) dendrimers are a class of water-soluble polymers that have demonstrated potential use as oral drug delivery
carriers (83). These macromolecular structures have a unique architecture, in which branching occurs with an exponential increase in the number of amidoamine groups. Each consecutive series of the branching step is termed a generation (G) and each increase in generation results in a concomitant increase in dendrimer size and number of surface groups. In general, “full generation” dendrimers are terminated with amine surface groups (e.g. G1, G2, etc.) and “half generation” dendrimers are terminated with carboxylate groups (e.g. G1.5, G2.5, etc.) (84). The branching multiplicity of dendrimers allows for therapeutic agents to be encapsulated in the interior void spaces, or conjugated to their surface groups (79). Importantly, PAMAM dendrimers are capable of traversing the intestinal epithelial barrier (84-88) and may allow a drug to bypass the P-gp efflux system (87), thereby rendering the dendrimer system uniquely suitable for applications in oral drug delivery. The molecular shape varies a lot between the individual generations of dendrimers, with lower generations (G4 and below) having ellipsoidal shapes and open structures, while G5 and above have a spherical shape and closed-shell structure (89). Unlike the classical polymerization which produces linear polymers of different sizes, dendrimers can be specifically controlled during the synthesis. Due to the high surface charge, these molecules have an exceptionally high solubility. Dendrimers being tightly packed balls in solution, have a lower viscosity than highly flexible linear polymers (90, 91). Attaching molecules to the dendrimers can be accomplished by trapping small molecules inside the ‘dendritic box’, and reacting the terminal amine group with an amino-acid. The trapped molecule is released from the box by hydrolyzing the head groups later (92). The general mechanism of the chain elongation of dendrimers is illustrated in Figure 1.3. Interestingly, PAMAM dendrimers have been shown to be
capable of traversing the intestinal epithelial barrier, thereby rendering the dendrimer system uniquely suitable for applications in oral drug delivery \((85, 87, 88)\).

\[
\begin{align*}
\text{Initiator Core} & \quad \text{G0} & \quad \text{G1} & \quad \text{G2} \\
\end{align*}
\]

**FIGURE 1.3:** Various generations of dendrimers and chain elongation.

### 1.4.3 Physiological role

In general, all cell-penetrating macromolecules are cationic at physiologic pH \((21)\). From physicochemical point of view, it is intriguing that these positively charged macromolecules cross the hydrophobic core of the lipid bilayer. Like with cell-penetrating peptides such as TAT-PTD and oligoarginine, the exact mechanism of entry of dendrimers is yet to be established beyond a reasonable doubt \((13)\). Several theories like adsorptive endocytosis \((12, 93)\) and inverted-micelle formation \((94)\) were proposed by several authors. The major forces driving the interaction in cell-penetrating system is proposed to be both electrostatic and hydrophobic with a concomitant osmotic imbalance \((78, 95)\). However, there is a considerable variation in the molecular structure and shape of these cell-penetrating macromolecules, which means that there may not be one single mechanism of action for all these molecules \((21)\). With potential applications, one has to bear in mind that leakage studies by several authors showed an increasing membrane disruption with higher generation dendrimers \((78, 93, 96)\).

### 1.5 Specific project aims
The mechanism of transport of proteins and peptides across the biological membranes is one of the most challenging problems in biochemistry and molecular biology. While cell cultures, tissues, and whole organisms are good systems to show the action of CPMs in conditions close to in vivo, the use of a simple, well-defined and well-understood model system provides better opportunity to reveal molecular mechanism of protein transduction. Regardless of the mechanism of translocation, the first necessary step must be binding of the CPM to the surface of the lipid membrane, and this is the topic of the present work. Based on our understanding of the interaction of proteins with biological membranes and surfaces, we plan to formulate an acceptable model for the possible mechanism of the interaction between two model CPMs (TAT-PTD and PAMAM dendrimers) and the lipid bilayer. We plan to accomplish this goal by the following:

We will study the association of TAT-PTD with the lipid bilayer as a function of

   a) lipid composition: neutral vs. acidic lipids, saturated vs. unsaturated lipids, phospholipids vs. glycolipids;
   b) transmembrane electric potential;
   c) physical state of the membrane: gel vs. liquid crystal;
   d) the presence of high salt, polycations, polyanions
   e) the effect of glycosaminoglycans such as: heparan sulfate, dermatan sulfate and low molecular weight heparin.

The data will be cross-checked and verified by simultaneous use of multiple techniques, such as fluorescence steady-state measurements, fluorescence quenching,
fluorescence anisotropy, and fluorescence resonance energy transfer. The macromolecular association will be described in suitable physico-chemical terms, such as binding constants or partition coefficients. The association will be analyzed quantitatively for the surface density of the peptide on the lipid bilayer and the glycoprotein.

The data will be quantitatively analyzed to give an insight into the percentage transfer of peptide through the lipid bilayer.

After getting an insight in the mechanism of interaction of TAT-PTD with model membranes, we will test synthetic dendrimers to compare the similarity or dissimilarity of interaction among the dendrimers and the TAT-PTD peptide, seeking to formulate a common mechanism of action, if there is one. In our present research in in-vitro conditions we will study variables, such as the presence of anionic lipids in the membrane, the presences of salt and polyions in the external environment, membrane fluidity, surface pressure, etc., which are largely difficult to control in biological systems.
CHAPTER 2
Materials and Methods

2.1 Materials

A fluorescent analog of TAT-PTD with a tyrosine replaced by tryptophan (H3N+-WGRKKRRQRRRPPQ-COO'), > 95% pure, was obtained from New England Peptide, Inc (Gardner, MA). N-acetyl tryptophanamide (NATA), and the lipid egg PC (L-α-phosphatidylcholine) were obtained from Sigma-Aldrich (St. Louis, MO). Lipids egg PG (L- α-phosphatidylglycerol), DPPS (dipalmitoyl phosphatidylserine ), DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine, sodium salt), DMPG (1,2-dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol)), sodium salt), were obtained from Avanti Polar Lipids (Alabaster, AL). Structure of the lipids is illustrated in Figure 2.1. The ionophore valinomycin and the fluorescence probes DiSC3(5) (3,3'-dipropylthiadicarbocyanine iodide), TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene), DPH (1,6-diphenyl-1,3,5-hexatriene), and pyrene-PG (1-hexadecanoyl-2-(1-pyrene-decanoyl)-sn-glycero-3-phosphoglycerol) were obtained from Invitrogen-Molecular Probes (Carlsbad, CA). Glycosaminoglycans: Heparan sulfate, fraction 1, sodium salt (from porcine intestinal mucosa, average molecular weight 13,655, sulfur content 5.51%); Heparin sodium, low molecular weight (from porcine intestinal mucosa, average molecular weight 4,494, sulfur content 10.4% ); Dermatan sulfate, sodium salt (from porcine intestinal mucosa, average molecular weight 41400, sulfur content 6.85%) were obtained from Celsus laboratories Inc. (Cincinnato, Ohio). All buffers and salts were from either Fisher Scientific (Pittsburgh, PA) or VWR (Atlanta, GA) and used at highest
purity available. Buffers were prepared containing 10 mM HEPES and 150 mM NaCl (for NaCl-HEPES buffer) or 150 mM KCl (for KCl-HEPES buffer), pH 7.4.

A)  

B)  

FIGURE 2.1: General structure of phospholipids; (A) phosphatidyl glycerol, (B) phosphatidyl choline.

For experiments with dendrimers, unlabelled monodisperse G1 (M.W. 1428 Da) and G4 (M.W. 14215 Da) PAMAM dendrimers, fluorescein isothiocyanate (FITC), Oregon-green were obtained from Sigma-Aldrich (St. Louis, MO) and were provided by Dr. Hamid Ghandehari of the University of Maryland-Baltimore. All other chemicals needed were used and obtained as mentioned above. Figure 2.2 illustrates the structure of PAMAM dendrimers and Figure 1.3, their generation sequence. In addition, Rhodamine-PE (1,2-dioleoyl-syn-glycero-3-phosphoethanolamine-n-(lissamine rhodamine B sulfonyl), were obtained from Avanti Polar Lipids (Alabaster, AL) and calcein was obtained from Invitrogen-Molecular Probes (Carlsbad, CA). Cobalt bromide (CoBr₂) obtained from Research Organic/Inorganic Chemical Co. (Sun Valley, CA.).

FIGURE 2.2: General structure of PAMAM dendrimer.
2.2 Model Membrane preparation

In our experiments we used small and large unilamellar vesicles (SUV and LUV, respectively) as model membranes. They were prepared according to the procedure described below.

2.2.1 Preparation of SUV

Small unilamellar vesicles (SUV) were prepared by sonication (97). Desired amounts of lipids were dissolved in chloroform and dried under a stream of nitrogen. The resulting dry thin film was hydrated in 0.5 ml buffer, so that the stock concentration of lipid was 13 mM. This lipid suspension was sonicated for 30 min at 4 °C (acidic lipids were sonicated shorter) with Sonic Dismembrator-Model 300 from Fisher Scientific. Fluorescently labeled SUV were prepared in the same way except that they contained the label (TMADPH, DPH or any other fluorophore) at a desired concentration.

2.2.2 Preparation of LUV

Large unilamellar vesicles (LUV) were obtained by extrusion, using LiposoFast extruder (Avestin, Inc., Ottawa, Ontario), after (98). The same lipid suspension as the one used for preparation of SUV was vortexed, frozen and thawed five times and extruded 21 times through stacks of the two polycarbonate membranes (Whatman Nucleopore, Clifton, NJ) with a pore diameter of 100 nm.
2. 2.3 Preparation of calcein-loaded SUV

For calcein-loaded SUV, calcein (Figure 2.3) was dissolved in distilled water and pH adjusted to 7.4. Concentration of calcein was calculated to be 80 mM in solution that was used to hydrate the lipid film prior to sonication as described above. The unentrapped calcein was removed by passing the SUV suspension (0.1 mL) through a Sephadex G-25 column (1 cm × 30 cm), eluted with NaCl-HEPES buffer as described above.

![Structure of calcein](image.png)

FIGURE 2.3: Structure of calcein.

2. 3 Determination of Concentrations

Absorbance of protein solutions in the range between 0.1 and 0.7 is a linear function of concentration. Concentrations were determined using Lamber-Beer's law

\[ A = \varepsilon b c \]  

where \( A \) is the absorbance of a particular fluorophore at a given wavelength, \( \varepsilon \) is the molar extinction coefficient in \( \text{cm}^{-1}/\text{M} \), \( b \) is the length of the light path (usually 1 cm), and \( c \) is the concentration of the solution in M.
Concentrations of dissolved TAT-PTD and other fluorophores was determined from absorption spectra using a JASCO V-530 UV spectrophotometer (Easton, MD) and Lambert-Beer law. Values of molar extinction coefficients used in the calculations are in Table 1 (13, 75, 88, 97).

The same spectrophotometer was used to record absorption spectra of fluorescently labeled lipid vesicles, used in FRET measurements.

2.4 Fluorescence Spectroscopy

2.4.1 Steady-state measurements

![Jablonski diagram illustrating the principle of fluorescence.](image)

The general principle of fluorescence is illustrated in Figure 2.4. Fluorescence measurements were performed with an ISS K2 fluorometer (Champaign, IL), equipped with a xenon lamp, variable slits, and a microprocessor-controlled photomultiplier. The
samples were measured in 1 cm x 1 cm quartz cuvettes. The excitation and emission wavelengths were 280 nm and 360 nm for tryptophan, respectively, 645 nm and 675 nm for DiSC3(5), 340 nm and 400 nm (monomer) or 475 nm (excimer) for pyrene, and 355 nm and 430 nm for TMADPH and DPH. When excitation was in the UV region, a 305 nm filter placed on the emission side was used to reduce light scattering from the lipid. Figure 2.5 shows the outlay of the fluorescence instrumentation.

![Fluorometer diagram](image)

**FIGURE 2.5:** General outlay of the Fluorometer.

Titrations of TAT-PTD with SUV, carried out at room temperature, were repeated 5 times. The data were corrected for dilution and analyzed using Microcal Origin 7.0 (Microcal Software, Northampton, MA). Binding of TAT-PTD to the lipid vesicles was evaluated from the decrease of fluorescence intensity at 360 nm. The data were fitted with double hyperbola using Eq. 2:

\[ F = 1 - \frac{f_1 x}{(K_{d1} + x)} - \frac{f_2 x}{(K_{d2} + x)}, \]

(2)
where \( F \) is the fluorescence intensity, normalized to 1 in the absence of lipid, \( x \) is the concentration of lipid, \( f_1 \) is the fraction of binding with the apparent dissociation constant \( K_{d1} \) and \( f_2 \) is the fraction of binding with the apparent dissociation constant \( K_{d2} \).

To measure the TAT-PTD-induced aggregation of negatively charged lipids in the membrane, SUV were prepared with 3\% pyrene-PG in DMPC/DMPG 3:1. Pyrene fluorescence was measured in the presence and absence of 8 \( \mu \)M TAT-PTD at an excitation wavelength of 340 nm. The ratio of intensities at 475 nm and 397 nm was taken as the excimer-to-monomer ratio.

To measure the effects of membrane fluidity, SUV were prepared with DMPC/DMPG at the ratio of 3:1. Titrations with TAT-PTD were carried out at 7°C, 17°C, 27°C, and 37°C. The temperature was maintained within 0.1 °C, using the circulating bath Neslab RTE -111 (Thermomeslab, Portsmouth, NH). Binding of TAT-PTD to the lipid vesicles was evaluated from fluorescence intensity at 360 nm.

2. 4.2 Fluorescence anisotropy

Fluorescence anisotropy was measured with the same fluorometer in the L format with the Glan-Thomson prism polarizers placed in the excitation and emission path. Data were collected in 5 independent measurements, each with 30 determinations.

2. 4.3 Fluorescence resonance energy transfer

Fluorescence Resonance Energy Transfer (FRET) was also measured with the same fluorometer. TAT-PTD was titrated with SUV labeled with 2\% TMADPH or DPH.
The efficiency $E$ of energy transfer between the two fluorophores was calculated using Eq. 3 (99):

$$E = \frac{A_{D}(\lambda_{D})}{A_{D}(\lambda_{D})} \cdot \left[I_{AD}(\lambda_{D}) / I_{A}(\lambda_{D}) - 1\right].$$

(3)

where $A_{D}(\lambda_{D})$ is the absorbance of the acceptor at the donor excitation wavelength, $A_{D}(\lambda_{D})$ is the absorbance of the donor at its excitation wavelength, $I_{AD}(\lambda_{D})$ is fluorescence intensity of the acceptor excited at the donor wavelength in the presence of the donor, and $I_{A}(\lambda_{D})$ is fluorescence intensity of the acceptor excited at the donor wavelength in the absence of the donor. The distance $R$ between the donor-acceptor pair was calculated from the efficiency $E$ using Eq. 4 (99)

$$R = R_{0} \cdot (1/E - 1)^{1/6},$$

(4)

where $R_{0}$, the Forster radius, is the distance between the donor and acceptor at which there is a 50% transfer of energy (i.e., $E = 0.5$).

### 2. 4.4 Fluorescence Quenching

TAT-PTD was maintained at 2 μM in the NaCl-HEPES buffer. Later 9.6 μM LUV or SUV (separately) with 25% negatively charged lipid were added and tryptophan fluorescence was measured at increasing concentrations of acrylamide (from 0.02 M to 0.51 M). The same set of experiments was also carried out with NATA, a soluble derivative of tryptophan, as a control. The data were analyzed using the Stern-Volmer equation (100), Eq. 4, with the intrinsic protein fluorescence multiplied by the factor $10^{[Q]}$ to correct for the acrylamide inner filter effect, using an extinction coefficient $\varepsilon$ of 4.3 cm$^{-1}$/M for acrylamide at 280 nm (101):

$$\frac{F_{0}}{F} = 1 + K_{SV} [Q]$$

(5)
where, $F_0/F$ is the ratio of unquenched and quenched fluorescence intensities, $[Q]$ is the molar concentration of the quencher and $K_{SV}$ is Stern-Volmer constant.

### 2.5 Membrane-Potential Dissipation

LUV were prepared with the KCl-HEPES buffer and stored in the same manner described earlier. An aliquot of the LUV stock (the final concentration of 4 μM) was placed in the NaCl-HEPES buffer and 250 nM DiSC$_3$(5) was added. DiSC$_3$(5) is a slow-reponse membrane potential probe, whose fluorescence is quenched in the presence of membrane potential (102). The potassium diffusion potential (negative inside) across the membrane was formed when the potassium ionophore valinomycin (2.25 nM; structure in Figure 2.6) was added to the lipid suspension. Values of the membrane potential were adjusted as needed by additions of the potassium buffer to the extravesicular environment and calculated from the Nernst equation (Eq. 6) (103):
\[ \Delta \psi = \frac{RT}{ZF} \ln \frac{C_{in}}{C_{out}}, \]  

(6)

where \( \Delta \psi \) is the membrane potential, \( R \) is the gas constant, \( T \) is the absolute temperature, \( C_{in} \) and \( C_{out} \) are the concentrations of ions potassium, \( Z \) is the charge or valence of the transported ion, and \( F \) is the Faraday constant. The derivation of this equation is discussed under section 3.3 in a more elaborate manner.

2.6 Glycosaminoglycan studies

A) HEPARAN (Heparitin) SULFATE

B) Repeat unit of heparin

C) Repeat unit of heparin

FIGURE 2.7: Structure of various glycosaminoglycans. (A) Heparan sulfate, (B) Chondroitin sulfate B/dermatan sulfate, (C) Low molecular weight heparin.
Structures of the three glycosaminoglycans (104) are shown in Figure 2.7. Fluorescence anisotropy changes of the tryptophan were quantified as a function of the glycosaminoglycan concentration. Fluorescence anisotropy was measured in the similar method as described under 2.4.2. The data were fitted using the Eq 7:

\[
\Delta f = \left[ \frac{f_{\text{max}} \cdot x}{K_d + x} \right] + f_{\text{init}}
\]

where \(\Delta f\) is the fluorescence anisotropy, \(x\) is the concentration of glycosaminoglycan, \(f_{\text{max}}\) is the maximum change in fluorescence anisotropy, at the saturating concentration of glycosaminoglycans, \(K_d\) is the apparent equilibrium dissociation constant and \(f_{\text{init}}\) is the initial fluorescence anisotropy. Lipid titration studies with TAT-PTD in the presence of glycosaminoglycans were carried out and the data were fitted using Eq 2.

2.7 Studies with PAMAM dendrimers

2.7.1 Fluorescence labeling of PAMAM dendrimers

The labeling of PAMAM dendrimers was done by Kelly Kitchens in Dr. Ghandehari’s laboratory at the University of Maryland-Baltimore (88). G1 and G4 dendrimers were conjugated to FITC or Oregon-green at a feed molar ratio of 1:1. Fluorescently labeled PAMAM dendrimers were purified by dialysis against distilled water using dialysis membranes of 500 MWCO (Spectrum Laboratories, Inc., Rancho Dominguez, CA). They were then fractionated on a Superose 12 HR 16/50 preparative column using a Fast Protein Liquid Chromatography (FPLC) system (Amersham Pharmacia Biotech, Uppsala, Sweden) with a mobile phase of 30%/70% (v/v) acetonitrile:Tris buffer (pH 8.0) at a flow rate of 1.0 ml/min. Fractions corresponding to
the appropriate dendrimer size and molecular weight were collected, dialyzed against distilled water, and lyophilized. The extent of FITC labeling was measured using the UV/Vis spectrophotometer at wavelength 490 nm.

2.7.2 Calcein release assay

Membrane integrity was assessed using leakage calcein, a self-quenching dye, which was entrapped in the vesicles at high concentrations. At constant concentration of lipid (35 µM), increasing concentrations of dendrimer were added and the steady state fluorescence measured. Percentage of the dye leakage was calculated using Eq. 8:

\[
% \text{ release} = \frac{[F-F_0]}{(F_{\text{max}} - F_0)} \times 100
\]

(8)

where \(F_0\) is the initial fluorescence intensity of calcein-loaded SUV, \(F\) is the fluorescence intensity after addition of dendrimers, and \(F_{\text{max}}\) is the fluorescence intensity after adding 20 µM triton.

2.7.3 Data analysis for dendrimers experiments

The experiments were performed and data were curve fitted using the same equations as those used for TAT-PTD. Changes employed for dendrimer experiments are mentioned below.
2.7.3.1 Fluorescence anisotropy studies with dendrimers

Anisotropy changes in the fluorescently labeled-dendrimer were quantified as a function of lipid concentration. Fluorescence anisotropy was measured by the method described under 2.4.2. The data were fitted using Eq 7.

2.7.3.2 Fluorescence quenching of labeled dendrimers

The FITC-Gldendrimer dissolved at 200 nM in the NaCl-HEPES buffer, 100 μM of 25 % PG SUV were added, and fluorescence intensity of FITC was measured at increasing concentrations of the quencher CoBr₂ (up to 0.64 mM). The same set of experiments was also carried out with 40 nM Oregon-green-G₄ dendrimer. The data were analyzed using the classical Stern-Volmer formalism or, when needed, using the equation modified for two quenching constants, Eq. 9:

\[
\frac{F}{F_0} = f_1/(1 + K_{SV1} [Q]) + (1-f_1)/(1 + K_{SV2} [Q])
\]

where, \( F/F_0 \) is the ratio of quenched and unquenched fluorescence intensities, \([Q]\) is the molar concentration of the quencher and \( K_{SV1} \) is the Stern-Volmer constant of a fraction of fluorophores \( f_1 \) and \( K_{SV2} \) is the second Stern-Volmer constant for the remaining fraction.

2.8 Surface tensiometry

Surface pressure of the lipid monolayer at the air-water interface is the difference between two surface tensions: that of water and that of the monolayer. Surface pressure was measured by a Langmuir trough Kibron μTrough S (Helsinki, Finland).
The molecular density at the air-water interface is manipulated with moveable Polytetrafluorethylene (PTFE or Teflon) barriers in a lipid film. The water subphase is temperature controlled. By moving PTFE barriers, the film density decreases (compression) or increases (expansion), which changes the surface tension $\gamma(LV)$ of the air-water interface. The quasi two-dimensional lateral or surface pressure $\Pi$ of the water surface can be calculated according to Eq. 10:

$$\Pi = \gamma_0(H_2O) - \gamma(LV)$$

(10)

As a reference, the surface tension of the pure water with $\gamma_0(H_2O) \approx 73$ mN/m at 20°C is chosen. Plots of the surface pressure $\Pi$ versus the molecular area of the amphiphiles (figure 2.8) at constant temperature $T$ (isotherms) are characteristic for the chosen amphiphile. The plot shows the surface isotherm for DMPE at 20°C.
At high molecular areas the monolayer exhibit a quasi two-dimensional gas state. The pressure increases at 78 Å²/molecule with further film compression into the liquid-expanded (LE) phase, where the amphiphiles are in contact without molecular ordering. The plateau at $\Pi_c = 5$ mN/m is a phase transition from the LE-phase to the liquid-condensed (LC) phase. In the LC-phase DMPE exhibit a short-range translational order of the headgroup positions. Simultaneously, the alkyl chains of the hydrophobic molecular part show a long-range order in their orientation, as indicated in the insets of for DMPE at 20°C (105, 106).

The lipid DMPE (1,2-Dimyristoyl-sn-glycero-phosphoethanol- amine) exhibits different phases as a monomolecular film (monolayer) at the air-water interface. The plateau hints at a phase transition from a non-ordered liquid-expanded (LE) phase to an ordered liquid-condensed (LC) phase. The red line indicates the pressure regime, where the DMPE monolayer is condensed during very slow transfer onto a hydrophilic silicon substrate.

HPLC-grade water was used to prepare buffers for surface pressure experiments. The stock concentrations of egg-PC and egg-PG used in these experiments were diluted to 2.5 mg of lipid per ml of chloroform. The glass plate was initially filled with 20 ml of HEPES-NaCl buffer and 0.5 µL of egg PC (for the electrically neutral monolayer), and 0.37 µL of egg PC and 0.13 µL of egg PG (for the monolayer with 25% negatively charged lipid) was placed on the surface of the buffer using a Hamilton syringe. The lipid molecules spontaneously spread on the surface and eventually occupy the air water interface with the polar head groups facing the water and the hydrophobic acyl chains.
sticking up into the air. The surface pressure during the experiments and graphs thereafter were obtained using Filmware 2.51 software, provided by the company. The dendrimer was added into the subphase-buffer using the Hamilton syringe in the increments of 2 μM. The experiment was terminated when no further increase in the surface was noticed, the usual final concentrations of dendrimer being 8 μM to 10 μM.

The fractional increase in the surface pressure ($P_d$) was reported using Eq. 11:

$$P_d = (P_f - P_i)/P_i,$$

where $P_f$ and $P_i$ are initial and final pressure attained at the end of the experiment, respectively.
CHAPTER 3

Interaction of TAT-PTD with Model Membranes

3.1 Introduction

Regardless of the controversy in the mechanism of interaction of TAT-PTD, the first essential step must be the binding of TAT-PTD to the lipid bilayer. We studied the interaction of TAT-PTD with model lipid membrane as a function of the negative charge density. The peptide, cationic at physiologic pH (7.4) would be expected to have a stronger interaction with model membranes containing anionic lipids. To test this hypothesis, fixed concentration of TAT-PTD was titrated with lipid membranes containing varying percentage of anionic lipids. The results were quantitated and evaluated within the framework of simple hyperbolic binding. Further, the preferential ability to aggregate anionic lipid by the cationic lipid was studied using the pyrene-labeled SUV. Pyrene-PG was used because the fluorophore pyrene has a high propensity for forming excimers (excited-state dimers), provided two pyrene monomers are sufficiently close to each other and in suitable orientation (107). Excimers decay to the ground state just as excited-state monomers do, but the excimers energy levels are lower than those of monomers. Consequently, fluorescence of excimers is significantly shifted towards longer wavelengths (lower energies) compared with that of monomers (Figure 3.1). Emission spectrum of pyrene, having two distinct peaks (monomer and excimer), can thus be used as an indicator of aggregation (or close proximity of pyrene molecules to each other) (107).
3.2 Results of titration of TAT-PTD with lipid

Titration of TAT-PTD with pure PC SUV resulted in a gradual decrease in the peptide's fluorescence (Figure 3.2). The peak intensity at 360 nm was taken and used to quantify the binding (Figure 3.3). Curve fitting with Eq. 2 revealed that double hyperbola fits the data the best (reduced chi-square value being 0.00011 for 0%, 0.00059 for 10%, 0.00144 for 25% negative charge; chi-square values for single-hyperbola fits were an order of magnitude greater). For pure PC SUV, two apparent dissociation constants were observed: $K_{d1} = 2.6 \pm 0.6 \mu M$ accounted for 24% of the extent of binding and $K_{d2} = 610 \pm 150 \mu M$ accounted for 76% of the interaction. When the titration was repeated with SUV containing 10% negatively charged lipid egg-PG, the values of $K_{d1}$ and the two fractions remained unchanged, but the value of the second dissociation constant $K_{d2}$ decreased to $420 \pm 30 \mu M$. Increasing the concentration of egg PG in the membrane to 25% resulted in a further decrease in $K_{d2}$ to $130 \pm 30 \mu M$. The value of $K_{d2}$ decreased linearly with increasing density of negative charge in the membrane (Figure 3.3, inset). No lipid-
induced shift in the tryptophan fluorescence spectrum was observed in either of the
titrations.

![Fluorescence Intensity vs Wavelength](image1.png)

**FIGURE 3.2:** Titration of TAT-PTD with SUV (PG - 0%)

![Fluorescence Intensity vs Lipid Concentration](image2.png)

**FIGURE 3.3:** Titration of TAT-PTD with SUV with different content of negatively charged lipid. (○) 0% PG, (▲) 10% PG, and (●) 25% PG. Inset shows the apparent dissociation constant $K_d$ as a function of PG concentration (mole %).

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It is reasonable to assume that the electrostatic binding of TAT-PTD occurs via simultaneous binding to eight negative charges in the membrane. This interaction, which would effectively neutralize the TAT-PTD molecule, requires that eight negatively charged lipid molecules move to close proximity of each other. We tested whether indeed TAT-PTD induced selective aggregation of negatively charged lipid by including in the membrane PG labeled with pyrene (Figure 3.4). Pyrene aggregation is marked in the fluorescence spectrum by the appearance of a broad excimer emission peak at 475 nm. The fluorescence ratio F475/F397 is taken as a measure of the excimer/monomer concentration ratio. Figure 3.4 shows the spectra of DMPC/DMPG (3:1) SUV labeled with 3% pyrene-PG in the presence and absence of TAT-PTD. The presence of the peptide clearly caused a decrease in the pyrene monomer fluorescence (the region between 370 nm and 400 nm) and a concomitant increase in the excimer fluorescence (at around 475 nm). The ratio F475/F397 was 0.077 and 0.166 in the absence and presence of TAT-PTD, respectively. Thus, TAT-PTD binding caused a greater than 100% increase in the excimer/monomer ratio, which proves the selective aggregation of pyrene-PG upon TAT-PTD binding.

![Graph](image)

**FIGURE 3.4:** Interaction TAT-PTD with pyrene-PG/DMPC/DMPG (3:75:22) SUV. Solid line, lipid only; dashed line, lipid + TAT-PTD.

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3.3 Effect of salt

When the titration of TAT-PTD with 25% negatively charged lipid was performed in the presence of 2 M NaCl we noticed a lesser decrease in fluorescence, compared with a similar plot at a low salt concentration (Figure 3.5). But when the salt was added after the lipid the tryptophan fluorescence did not change (Figure 3.6).

FIGURE 3.5: The effect of 2 M NaCl on TAT-PTD binding. ($\Delta$) presence of salt, and (●) absence of salt.

FIGURE 3.6: The effect of 2 M NaCl on TAT-PTD binding – added at the end of the titration.
3. 4 Fluorescence anisotropy

Fluorescence anisotropy can be used to study binding between two molecules and to determine the binding constant (or the inverse, the dissociation constant) for the interaction. The basic idea is that a fluorophore excited by polarized light will also emit polarized light. However, if a molecule is moving during the lifetime of fluorescence excitation, the emitted fluorescence will be depolarized, or have lower anisotropy. The loss of anisotropy is greatest with fluorophores freely rotating in solution and decreases with decrease in the freedom of rotation (108). Figure 3.7 illustrates the general scheme of fluorescence anisotropy. Protein interactions can be detected when one of the interacting partners is fused to a fluorophore. Upon binding of the partner molecule a larger, more stable complex is formed which will decrease the freedom of rotation, thus increasing the anisotropy of the emitted light.

![Diagram of fluorescence anisotropy](image)

FIGURE 3.7: General scheme of the principle of fluorescence anisotropy.

Anisotropy measurements are based on the principle of photoselective excitation of fluorophores by polarized light. Fluorophores preferentially absorb photons whose electric vectors are aligned parallel to the transition moment of the fluorophore. The transition moment has a defined orientation with respect to the molecular axis. In an
isotropic solution, the fluorophores are oriented randomly. Upon excitation with polarized light, one selectively excites those fluorophore molecules whose absorption transition dipole is parallel to the electric vector of the excitation. This selective excitation results in partially polarized fluorescence emission. Emission also occurs with the light polarized along a fixed axis in the fluorophore. The relative angle between these moments determines the maximum measured anisotropy. The fluorescence anisotropy $r$ is defined as

$$ r = (I_\parallel - I_\perp) / (I_\parallel + I_\perp) $$

where $I_\parallel$ and $I_\perp$ are the fluorescence intensities of the vertically and horizontally polarized emission, when sample is excited with vertically polarized light.

Fluorescence anisotropy of TAT-PTD was measured in the presence and absence of the lipid (Figure 3.8). The steady-state anisotropy of the TAT-PTD tryptophan fluorescence in the buffer was $-0.008 \pm 0.002$. Upon binding to SUV made of a neutral lipid, the value increased to $0.045 \pm 0.002$, whereas with SUV that included 25% negatively charged lipid the anisotropy increased to $0.088 \pm 0.002$.

![Fluorescence anisotropy change upon interaction of TAT-PTD with different lipids. (○) 0% PG, and (●), 25% PG.](image)
3.5 Effect of the lipid phase state

Fluidity of the membrane depends on temperature (thermotropic phase transitions) and the lipid composition (lyotropic phase transitions) of the membrane. Subsequent experiments were designed to study how the lipid phase state, or membrane fluidity, influences binding of TAT-PTD to the membrane.

To that aim, we carried out titrations of TAT-PTD with SUV prepared with DMPC/DMPG 3:1. Chemically defined lipids DMPC and DMPG were used instead of egg-PC and egg-PG because the former lipids have a sharp phase-transition temperature between the gel and liquid-crystal states. Below 23°C these lipids are in the gel state, characterized by low fluidity and slow lateral and rotational diffusion, and above that temperature they are in the liquid-crystal state, marked with a high fluidity and fast diffusion (109, 110). The lipid-induced changes in TAT-PTD fluorescence greatly increased at higher temperatures, when the membrane was fluid, as reflected in the greater fluorescence change (Figure 3.9).

FIGURE 3.9: (A) Titration of TAT-PTD with 3:1 DMPC/DMPG at temperatures 7°C (Δ), 17°C (○), 27°C (★), and 37°C (□). (B) Effect of temperature on the TAT-PTD binding to DMPC/DMPG SUV.

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3.6 Membrane Potential Studies

Normal cells in the living system maintain an electric potential difference across the membrane, which is termed 'the membrane potential' (32). Membrane potential is created because of the imbalance between the amount of ionic charges inside and outside the cell. Membrane potential is an electrochemical term, which represents the free energy involved in moving a mole of ions between two phases separated by the membrane. This potential $\mu$ across the membrane is given by the Nernst equation, which is easily derived from the expression for electrochemical potential in two phases:

$$\mu(\text{out}) = RT \ln C(\text{out}) + ZF \psi(\text{out})$$ \hspace{1cm} (12.1).

$$\mu(\text{in}) = RT \ln C(\text{in}) + ZF \psi(\text{in})$$ \hspace{1cm} (12.2).

where $R$ is the gas constant; $T$ is the absolute temperature; $C$ is the ion concentration; $Z$ is the charge or valence of the transported ion; $F$ is the Faraday constant; and $\psi$ is the electric potential. The electrochemical potential difference between the two phases is

$$\Delta\mu = \mu(\text{in}) - \mu(\text{out}) = [RT \ln C(\text{in}) + ZF \psi(\text{in})] - [RT \ln C(\text{out}) + ZF \psi(\text{out})]$$ \hspace{1cm} (12.3)

When simplified:

$$\Delta\mu = RT \ln \frac{C(\text{in})}{C(\text{out})} - ZF \psi$$ \hspace{1cm} (12.4)

$$\Delta\mu = RT \ln \frac{C(\text{in})}{C(\text{out})} - ZF \Delta\psi$$ \hspace{1cm} (12.5)

At equilibrium $\Delta\mu$ should be equal to zero, and therefore

$$ZF \Delta\psi = RT \ln \frac{C(\text{in})}{C(\text{out})}$$ \hspace{1cm} (12.6) or

$$\Delta\psi = \frac{RT}{ZF} \ln \frac{C(\text{in})}{C(\text{out})}$$ \hspace{1cm} (12.7)

$\Delta\psi$ is called membrane potential and Eq.12.7 the Nernst equation. With all the constants substituted, the equation acquires the following form:

$$\Delta\psi \sim -59 \log \frac{C(\text{in})}{C(\text{out})} \text{ Volts}$$ \hspace{1cm} (12.8).
Biomembranes are frequently held away from equilibrium by expenditure of metabolic energy. Ion pumps can maintain ionic concentration differences and hence generate membrane potential.

Membrane potential in our system was not created by metabolic ion pumps. Rather, we utilized the very low permeability of the lipid membrane to ions and, with the help of two different potassium ion concentrations on the two sides of the membrane and the potassium-specific ionophore valinomycin, which preferentially transfers potassium \([K^+]\) ions down its electrochemical potential gradient, we created a passive form of membrane potential, called diffusion potential. (It is created through facilitated diffusion of potassium ions across the membrane.)

Valinomycin is a cyclododecadepsipeptide ionophore antibiotic produced by *Streptomyces fulvissimus*. It is composed of three molecules each of L-Valine D-alpha-hydroxyisovaleric acid, D-Valine and L-lactic acid linked alternately to form a 36-membered ring. Valinomycin is a potassium-selective ionophore and is commonly used as a tool in biochemical studies. K+ ions bind to valinomycin and are shuttled back and forth across the membrane. In the absence of a potassium-specific ionophore like valinomycin, K+ only rarely crosses a lipid bilayer. In the presence of valinomycin, K+ is freely permeable.

We detected and measured membrane potential by employing potential-sensitive fluorescence probes. There are two types of such probes. Fast-response membrane-potential probes (Figure 3.10) operate by means of a change in their electronic structure, and consequently their fluorescence properties, in response to a change in the surrounding electric field. Their optical response is sufficiently fast to detect transient
potential changes in excitable cells, like neurons, cardiac etc. Fast response probes typically show a 2-10% fluorescence change per 100 mV. Slow response probes (Figure 3.10) show potential-dependent changes in their transmembrane distribution that are accompanied by fluorescence changes. The magnitude of their optical response is much larger than that of fast-response probes, which is typically ~1% fluorescence change per mV. Slow response probes (like DiSC₃(5), illustrated in Figure 3.11) may be used for studying membrane potential of nonexcitable cells, which is caused by respiratory activity, ion-channel permeability, drug binding, etc.

FIGURE 3.10: General outline of the mechanism of action of membrane potential dyes.

FIGURE 3.11: Structure of DiSC₃(5).
To study the effect of membrane potential on the interaction of TAT-PTD with lipid bilayer, we created an in vitro membrane potential as described in Materials and Methods. Experiments were carried out with both 0% and 25% PG LUV. In either experiments we noticed no dissipation of membrane potential upon addition of TAT-PTD (results with 25% PG LUV are shown in Figure 3.12). In another experiment we tested the possible effect of membrane potential on TAT-PTD binding to the membrane. We observed no changes in fluorescence spectra of the membrane-bound peptide upon formation of membrane potential (Figure 3.13).

![Graph](image1)

**FIGURE 3.12:** Effect of TAT-PTD on membrane potential (165 mV, negative inside) in 25% PG LUV.

![Graph](image2)

**FIGURE 3.13:** Fluorescence of tryptophan of TAT-PTD upon interaction with 25% PG SUV in the presence of membrane potential.
3.7 Localization of TAT-PTD with respect to the membrane

On order to determine localization of the peptide bound to the membrane, we employed fluorescence quenching, fluorescence anisotropy, and fluorescence resonance energy transfer.

3.7.1 Fluorescence quenching studies

![Acrylamide structure](image)

FIGURE 3.14: Structure of acrylamide.

The localization with respect to the membrane of the N-terminal tryptophan residue of the membrane-bound TAT-PTD can be inferred from fluorescence quenching data. Acrylamide (Figure 3.14) appeared to quench the TAT-PTD fluorescence completely both in solution and bound to the membrane (Figure 3.15A). Quenching of the water-soluble tryptophan derivative NATA showed exactly the same pattern (Figure 3.16). The values of Stern-Volmer quenching constant were $28 \pm 2 \text{ M}^{-1}$ and $32 \pm 3 \text{ M}^{-1}$ for TAT-PTD in the absence and presence of SUV with 25% PG, respectively.

Acrylamide can to some extent partition in nonpolar environments \cite{111}, therefore we repeated the quenching experiments with iodide, which, being an anion, selectively quenches fluorophores exposed to the aqueous phase. The results were the same as with
acrylamide (Figure 3.15B): KI completely quenched the tryptophan fluorescence both in the absence and presence of SUV with 25% PG. The values of Stern-Volmer quenching constants were $26 \pm 1 \text{ M}^{-1}$ and $29 \pm 1 \text{ M}^{-1}$, respectively.

**FIGURE 3.15:** (A) Fluorescence quenching of TAT-PTD with acrylamide, in the presence and absence of lipid. (B) Fluorescence quenching of TAT-PTD by potassium iodide. ○, absence of lipid; Δ, presence of lipid (25% PG).

**FIGURE 3.16:** Fluorescence quenching of NATA with acrylamide, in the (●)presence and (○)absence of 25% PG lipid.

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3.7.2 Fluorescence anisotropy studies with membrane probes

To study the possible peptide-induced changes in the lipid order in the bilayer during the peptide-lipid interaction, the membranes were labeled with the lipophilic probes DPH and TMA-DPH and the anisotropy of their fluorescence was measured. The relative position of TMA-DPH and DPH in the lipid membrane is shown in Figure 3.17. The values were $0.117 \pm 0.003$ and $0.225 \pm 0.004$ for DPH and TMA-DPH, respectively, and they did not change upon binding TAT-PTD ($0.122 \pm 0.003$ and $0.229 \pm 0.004$ respectively).

A)

B)

C)
3.7.3 Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic states of two fluorescent molecules in which the excitation wavelength of an acceptor molecule is within the emission band of the donor molecule (but the energy is transferred from the donor molecule to the acceptor molecule without emission of a photon). The efficiency of FRET is dependent on the inverse sixth power of the intermolecular separation, making it useful over distances comparable with the dimensions of biological phenomena that produce changes in molecular proximity. The distance at which energy transfer is 50% efficient (i.e., 50% of excited donors are deactivated by FRET) is defined by the Forster radius ($R_0$). The magnitude of the constant $R_0$ is only dependent on spectral properties of the donor and acceptor pair.

![Figure 3.17: A) Structure of DPH; B) Structure of TMA-DPH; C) Relative localization of TMA-DPH and DPH in the lipid membrane.](image)

![Figure 3.18: (A) Titration of TAT-PTD with 25% PG SUV labeled (○) and unlabeled (●) with 2% TMA-DPH. (B) FRET between tryptophan and TMA-DPH. Spectrum 1, TMA-DPH SUV excited at 280 nm; spectrum 2, after addition of TAT-PTD; spectrum 3, after subtraction of the peptide fluorescence; shaded area, energy transfer – after subtraction TMA-DPH fluorescence (spectrum 1) due to direct excitation of the probe with 280-nm light.](image)
When TAT-PTD was titrated with the lipid labeled with 2% TMA-DPH, a greater decrease in the tryptophan fluorescence was noticed than with the unlabelled lipid, as shown in Figure 3.18A. The additional quenching is attributed to energy transfer from tryptophan to TMA-DPH, which is more rigorously documented in figure 3.18B. These data allowed for calculation of the distance between the tryptophan of TAT-PTD and TMA-DPH, whose charged head group anchors the fluorophore moiety close to the membrane interface. Efficiency of energy transfer was determined using Eq. 3 and the distance between the two fluorophores was calculated using Eq. 4. We observed an energy transfer efficiency of 50% and therefore the distance between the two fluorophores is 3.4 nm, the value of \( R_0 \) for the tryptophan/TMA-DPH pair. In contrast with TMA-DPH, inclusion of 2% DPH into the membrane did not cause an additional decrease in the TAT-PTD fluorescence (Figure 3.19). Within the limits of sensitivity of our instrument, there was no appreciable energy transfer to DPH, which partitions deeper in the hydrophobic core of the lipid bilayer.

![Figure 3.19: Titration of TAT-PTD with 25% PG SUV labeled (□), and unlabeled (●) with 2% DPH.](image-url)
3.8 Effect of peptidoglycans

A complex formation between the cationic peptide and an anionic binding partner has been proposed as an alternative mechanism to reduce the effective electric charge of the peptide. Such a larger, but electrically neutral complex could conceivably cross the lipid membrane easier than a highly charged naked peptide. Some authors have hypothesized that glycosaminoglycans of the extracellular matrix, such as heparan sulfate, are needed for the transport of TAT-PTD peptide through the cell membrane (63-65). We employed the developed fluorescence techniques to study the binding between the negatively charged glycosaminoglycans and the positively charged peptide and the possible effect of such binding on the interaction of the peptide with the lipid bilayer.

3.8.1 Interaction of TAT-PTD with glycosaminoglycans

An increase in fluorescence anisotropy was noticed upon titration of TAT-PTD (concentration maintained constant at 3 μM) with all three glycosaminoglycans. The highest concentration of the individual glycosaminoglycan to limit the titration was varied depending on the fluorescence anisotropy change (Figure 3.20A, 3.20B, 3.20C). The curve fitting of the data using Eq. 7 resulted in the following values of apparent binding constants ($K_{dg}$), 8.9 ± 2.3 μM, 3.9 ± 0.5 μM, and 1.5 ± 0.5 μM for heparan sulfate, dermatan sulfate, and low-molecular-weight heparin, respectively. Steady-state emission spectra of tryptophan showed no changes either in fluorescence intensity or in the peak position during the interaction of TAT-PTD with all three glycosaminoglycans (data not shown).
3.8.2 Effect of salt

It is safe to assume the interaction between cationic TAT-PTD and anionic glycosaminoglycans are completely electrostatic. When 2 M sodium chloride was added at the end of titration (TAT-PTD with glycosaminoglycans) we noticed a complete restoration of tryptophan’s low fluorescence anisotropy (Figure 3.21). Furthermore, when the whole titration was carried out in the presence of salt (2M NaCl), no increase in anisotropy was observed at all (Figure 3.21). This firmly establishes a complete electrostatic nature of the interaction between TAT-PTD and glycosaminoglycans.
3.8.3 Titration of TAT-PTD with lipid in the presence of glycosaminoglycans

We have shown (3.2) that the binding affinity of TAT-PTD to small unilamellar vesicles (SUV) is linearly proportional with the amount of surface negative charge (on SUV) (13). Also, we noticed two apparent binding constants indicating two modes on binding. $K_{d1}$, the apparent dissociation constant for non-electrostatic component accounting for 24% of the interaction was found to be $2.6 \pm 0.6 \mu M$ and $K_{d2}$, the apparent dissociation constant for electrostatic component accounting for 76% of the interaction was found to be $130 \pm 30 \mu M$ (for 25% PG SUV) (13). In our present research, we kept the TAT-PTD and glycosaminoglycan concentrations constant (at 3 $\mu M$ and 100 $\mu M$, respectively) and followed the changes in the fluorescence intensity of tryptophan (peak intensity taken at 360 nm) upon titration with SUV prepared with 25% PG (Fig. 3.22). The concentration of glycosaminoglycans was kept high so that complete
saturation of TAT-PTD can be assumed. $K_{d1}$, the non-electrostatic component (accounting for 24% of the interaction) was found to be $1.7 \pm 0.3 \, \mu M$, $37 \pm 9 \, \mu M$, $5.7 \pm 0.7 \, \mu M$, and $K_{d2}$, the electrostatic component (accounting for 76% of the interaction) was found to be $1.14 \pm 0.07 \, \text{mM}$, $2.64 \pm 0.37 \, \text{mM}$, and $2.03 \pm 0.13 \, \text{mM}$ for heparan sulfate, dermatan sulfate, and low molecular weight heparin, respectively. It can be noticed that, in the presence of glycosaminoglycans there is a decrease in the affinity of interaction of TAT-PTD with 25% PG SUV. Furthermore, no blue shift in the tryptophan emission spectra was noticed upon interaction with SUV even in the presence of glycosaminoglycans (data not shown). It can be assumed that neutralization of the surface positive charge of TAT-PTD with glycosaminoglycans hinders its interaction with negatively charged SUV.

When the glycosaminoglycans were replaced with 2M NaCl and titration carried out, the apparent dissociation constants $K_{d1}$ and $K_{d2}$ were found to be $2.6 \pm 0.6 \, \mu M$ and $0.95 \pm 0.07 \, \text{mM}$, respectively. This goes on to prove that the polycations like glycosaminoglycans inhibit the interaction of TAT-PTD with SUV more than monovalent salt ions.

![Graph](image_url)

**FIGURE 3.22:** Interaction of TAT-PTD with 25% PG SUV in the presence of 0.1 mM glycosaminoglycans; (inverted Δ) no external anions, (half filled inverted Δ) 2M NaCl, (○) heparan sulfate, (Δ) dermatan sulfate, (○) low molecular weight heparin.

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CHAPTER 4

Interaction of PAMAM dendrimers with model membranes

4.1 Effect of Lipid Composition

Membrane interactions of the dendrimers was followed by changes in the anisotropy of the fluorophore on the labeled dendrimer. The fluorescence anisotropy of FITC-G1-dendrimer (200 nM) increased upon interaction with 0% PG and 25% PG SUV. Quantitative analysis of the data using Eq. 7 yielded the values of apparent dissociation constant ($K_d$), which were found to be $30 \pm 6 \mu M$ for 0% PG SUV and $11 \pm 3 \mu M$ for 25% PG SUV. In contrast with the FITC-labeled dendrimer, Oregon-green-labeled G4 dendrimer exhibited a decrease in the fluorescence anisotropy upon binding to the membrane. The decrease is assumed to be due to the difference in photophysical properties of the fluorophore. When the data were analyzed for binding constants we found $K_d$ for G4 dendrimer to be $16 \pm 7 \mu M$ with 0% PG SUV and $5 \pm 1 \mu M$ with 25% PG SUV (Figure 4.1A, 4.1B).

![Graphs showing fluorescence anisotropy vs. lipid concentration for FITC-G1 and Oregon-green-G4 dendrimers.](image)

**FIGURE 4.1:** (A) Interaction of FITC-G1 dendrimer with lipid; (B) Interaction of Oregon-green-G4 dendrimer with Lipid; (□) 0% PG SUV, and (■) 25% PG SUV.
4. 2 Effect of salt

The electrostatic nature of the dendrimer-membrane interactions can be obviously predicted due to the high charge on the surface of the dendrimers. We confirmed this prediction by salt titration studies. FITC-G1 dendrimer had anisotropy of 0.029 ± 0.016 (Figure 4.2A), which in the presence of two molar salt increased to 0.039 ± 0.007. When the negatively charged SUV (with 25% egg-PG) was added to the FITC-G1 dendrimer the anisotropy was 0.042 ± 0.006 and 0.091 ± 0.006 respectively, in the presence and absence of salt. However, when salt solution was added to the dendrimer-lipid suspension (making the final salt concentration two molar), the fluorescence anisotropy dropped back to 0.044 ± 0.004. The Oregon-green-G4 dendrimer also showed a difference in the lipid-induced anisotropy changes (Figure 4.2B) depending on the presence or absence of high salt. Fluorescence anisotropy of pure Oregon-green-G4 in buffer was 0.079 ± 0.005, and in the presence of two-molar salt it increased to 0.092 ± 0.012. The values of the
dendrimer-lipid suspension were 0.040 ± 0.006 and 0.091 ± 0.009 in the presence and absence of salt respectively, and when salt was added to the latter sample after the titration with lipid, the anisotropy was 0.081 ± 0.007. The consistent increase in the fluorescence anisotropy of both FITC-G1 and Oregon-green-G4 dendrimers in buffer caused by high salt concentrations (in the absence of lipid) is assumed to be due to the salt-induced change in dipole distribution within the fluorophore molecules.

4.3 Leakage Studies

![Graphs showing leakage studies](attachment:graph.png)

FIGURE 4.3: (A) Baseline to test for stability of calcein loaded SUV; (B), Release of calcein; (1) 7.4% release-G4 with 25% PG SUV, (2) 5.2% release-G1 with neutral SUV, (3) 3.4% release-G4 with 25% PG SUV, (4) 0% release-G1 with neutral SUV.

The membrane-disruptive properties of polycationic molecules were studied earlier using lipid-mixing and leakage studies (93). These authors showed a greater release of intravesicular fluorophore calcein with high generation (G5 and above) dendrimers. We studied the dendrimer-induced calcein leakage from the neutral and 25% negatively charged SUV with G1 and G4 dendrimers. The data were analyzed using Eq. 

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8. In our studies with low generation dendrimers, we noticed a 7.4% release of calcein with 25% PG SUV-G4 dendrimer interaction, 5.3% release of calcein with 0% PG SUV-G4 dendrimer interaction, 3.2% release with 25% PG SUV-G1 dendrimer interaction, and 0% release with 0% PG SUV-G1 dendrimer interaction (Figure 4.3 and 4.4).

![Graph](image)

FIGURE 4.4: (A) Titration of calcein SUV (0% PG) with increments of G1; (B), Titration of calcein SUV (25 % PG) with increments of G4.

### 4.4 Effect of the physical state of the membrane

Membrane fluidity and lateral phase separation during the dendrimer-lipid interaction was studied using 3% pyrene-PG label in 25% negatively charged SUV. The excimer/monomer ratio revealed a selective aggregation of anionic lipids induced by the polycationic dendrimer. The pyrene excimer/monomer ratio in the labeled lipid was 0.038 (lipid concentration 100 µM). When unlabeled G1 dendrimer (300 µM) was added the ratio increased to 0.111, and with unlabeled G4 it was found to be 0.214. These data show an approximately two and a half times increase in the ratio with G4 over G1 dendrimer (Figure 4.5). The observed increase in the pyrene excimer/monomer ratio

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indicates that the pyrene-labeled PG molecules get closer to each other upon the dendrimer binding to the membrane. The data was further analyzed to critically assess the relation between the excimer and the surface charge density of the dendrimer. The ability of the PG molecules to segregate might be influenced by membrane fluidity. To test the effect of membrane fluidity we let the dendrimers interact with the membranes of identical compositions but different physical state. studied the effect of gel and liquid-crystalline phase of lipid on the dendrimer-lipid interaction (Figure 4.6). To study this we used chemically defined lipids DMPC and DMPG which have a sharp phase transition temperature at 21-23 °C (109, 110). Below this temperature dimyristoyl phospholipids are in the gel state, with greatly limited translational diffusion in the membrane, and above this temperature they are in a fluid liquid-crystal state. Keeping the concentrations of dendrimers the same as in Figure 4.1, SUV prepared, two sets of experiments at 7 °C and 37 °C were performed with DMPC/DMPG (3:1) SUV. We observed that both FITC-G1 and Oregon-green-G4 dendrimers had higher binding affinity with lipids in the liquid-crystalline phase.
FIGURE 4.5: Interaction dendrimers with pyrene-PG/DMPC/DMPG (3:75:22) SUV. Spectrum 1, lipid only; spectrum 2, labeled lipid + G1 dendrimers; spectrum 3, labeled lipid + G4 dendrimers. Inset shows the excimer/monomer ratio as a function of the surface charge density.

FIGURE 4.6: (A) Effect of temperature on the interaction of FITC-G1 dendrimer with lipid; (B) Effect of temperature on the interaction of Oregon-green-G4 dendrimer with lipid. (▲) 3:1 DMPC/DMPG at 7 °C; (●) 3:1 DMPC/DMPG at 37 °C.

4.5 Localization of dendrimers with respect to the membrane

Localization of dendrimers with respect to the membrane, or the degree of insertion of dendrimers into the membrane, was studied by two techniques—fluorescence quenching and FRET. When FITC-G1 dendrimer was titrated with the negatively charged lipid labeled composed of egg phosphatidylcholine/phosphatidylglycerol/lissamine-rhodamine-phosphatidylethanolamine (73:25:2), a decrease in the intensity of FITC fluorescence spectrum is noticed. This quenching is due to energy transfer from FITC to rhodamine B (Figure 4.7). These data allowed for calculation of the distance between the two fluorophores, whose charged head group anchors it close to the membrane interface. Efficiency of energy transfer was determined using Eq. 3 and the distance between the two fluorophores was calculated using Eq. 4. We observed an energy transfer efficiency
of 60%, which in conjunction with the value of Ro = 5.4 nm (99), yields the distance between the two fluorophores 4.8 ± 0.3 nm. In contrast, with Oregon-green-G4 dendrimer, which is larger than G1 dendrimer, the energy transfer efficiency was found to be 99% and the measured distance was 2.0 ± 0.3 nm (Figure 4.8).

FIGURE 4.7: FRET between FITC-G1 dendrimer and rhodamine-labeled lipid showing 60% energy transfer. Spectrum 1, FITC-G1 dendrimer excited at 480 nm; spectrum 2, after addition of rhodamine-labeled SUV; spectrum 3, after subtraction of the normalized FITC-G1 dendrimer; spectrum 4, rhodamine-labeled SUV excited at 480 nm; spectrum 5, FRET showing 60% energy transfer.

FIGURE 4.8: FRET between Oregon-green-G4 dendrimer and rhodamine-labeled lipid. Spectrum 1, Oregon-green-G4 dendrimer excited at 480 nm; spectrum 2, after addition of rhodamine-labeled SUV; spectrum 3, after subtraction of the normalized Oregon-green-G4 dendrimer; spectrum 4, rhodamine-labeled SUV excited at 480 nm; spectrum 5, FRET showing 99% energy transfer.
The higher binding affinity of G4 dendrimer compared to G1 can be easily explained by the larger size and surface charges on G4. To get a better insight into the actual binding mechanism, using Co$^{2+}$ (97) as the quencher, we studied how fluorescence quenching of the two fluorophores changes in the presence of lipid (Figure 4.9). The data were later analyzed using the Eq. 5. The classical linear Stern-Volmer formalism could not be used to analyze our data. Introduction of a second quenching constants was necessary to explain the observed non-linear quenching, which indicates that there were two populations of fluorophores, one population of fluorophores easily amenable to quenching (having a lower quenching constant) and the second population more difficult to quench (having a higher quenching constant). With FITC-G1 dendrimer in the absence of lipid the quenching constants were found to be 12 ± 1 mM$^{-1}$ and 190 ± 70 mM$^{-1}$ with fractions 70% and 30%, respectively, and in the presence of lipid the quenching constants were 9 ± 2 mM$^{-1}$ and 280 ± 70 mM$^{-1}$ with fractions 37% and 63%, respectively. The weight-averaged quenching constants for FITC-G1 were calculated to be 65.4 ± 26.2 and 179.7 ± 58.6 in the presence and absence of lipid, respectively. In the similar set of experiments repeated with Oregon-green-G4 dendrimer (Figure 4.10) in the absence of lipid the quenching constants were found to be 8 ± 3 mM$^{-1}$ and 100 ± 70 mM$^{-1}$ with fractions 74% and 26%, respectively, and in the presence of lipid the quenching constants were 7 ± 1 mM$^{-1}$ and 230 ± 60 mM$^{-1}$ with fractions 48% and 52%, respectively. The weight-averaged quenching constants for Oregon-green-G4 were calculated to be 31.9 ± 16.8 and 122.9 ± 49.4 in the presence and absence of lipid, respectively. However,
FITC-G1 dendrimer quenching done in high salt external environment (1M NaCl) was found to follow a linear Stern-Volmer kinetics (Figure 4.9) with single quenching constant \( K_{SV} = 13 \pm 1 \text{ mM}^{-1} \). We have already seen the effect of high salt on dipole distribution within the FITC moiety. In dendrimers with high positive surface charge, complex intramolecular electrostatic interactions may play additional role and explain the two populations of fluorophores with different accessibilities to the quencher.

FIGURE 4.9: (A) Quenching of FITC-G1 dendrimer by cobalt bromide in the presence and absence of lipid; (B) Stern-Volmer Transformation of A, showing non-linear kinetics; (■) absence of lipid, (▲) presence of lipid, (●) presence of 1M NaCl.

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Figure 4.10: (A) Quenching of Oregon-Green G4 dendrimer by cobalt bromide in the presence and absence of lipid; (B) Stern-Volmer Transformation of A, showing non-linear kinetics; □ absence of lipid, ▲ presence of lipid.

4.6 Surface Pressure Studies
The effect of dendrimers on the lipid membrane, that is, either membrane penetration or membrane rupture, was tested using the Langmuir trough. The experiments were performed with both 0% and 25% PG SUV (Figure 4.11). In our experimental setup we found that an error of up to 3% (with $P_i$ 20 mN/m and 30 mN/m) and upto 10% (with $P_i$ 10 mN/m) was unavoidable, the probable reason being external air pressure disturbance on the sensor. The data interpretation was thus done accordingly. As it can be seen from Figure 4.12, there was always an increase in the surface pressure, and at lower $P_i$ (at 10 mN/m) there was more than 100% increase in the pressure, indicating the insertion of the dendrimers at lower pressures. However, when $P_i$ was maintained at 30 mN/m (physiologic surface pressure ranging 29-33 mN/m) (112), no major increase in surface pressure was observed with either of the dendrimers, suggesting a lack of insertion at that surface pressure.

![Graph](image)

**FIGURE 4.12:** Effect of dendrimers on the lipid monolayer. (■), G1 dendrimer with 0% PG SUV; (○), G1 dendrimer with 25% PG SUV; (▲), G4 dendrimer with 0% PG SUV; (▼) G4 dendrimer with 25% PG SUV.

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CHAPTER 5

Discussion and Conclusion

5.1 TAT-PTD

The mechanism of penetration of TAT-PTD through the lipid bilayer is not known, although current evidence favors endocytosis (6, 40, 113). Regardless of the nature of subsequent events, the first necessary step for TAT-PTD penetration must be binding to the surface of the membrane. We studied this initial step using biophysical techniques with the aim of gaining a quantitative insight into the process and using the model system of pure lipid vesicles, which has a limited number of variables and is better defined and controlled than live cells. The use of the model system has allowed us to characterize the membrane interaction of TAT-PTD as a function of physico-chemical properties of the lipid bilayer.

Even though there were some early reports raising the possibility of specific cell surface receptors for TAT protein (114, 115), experiments with L and D-enantiomeric peptides of the CPP penetratin (38) do not favor the idea of CPPs binding to a specific receptor. Indeed, the high positive charge on TAT-PTD almost predestines it to bind non-specifically to any negatively charged molecule or organelle, including the lipid membrane. Binding of TAT-PTD to glycosaminoglycans, components of extracellular matrix (63, 64, 104), has been considered biologically relevant for CPPs binding to, and penetration across, the cell membrane, but our data as well as recent data from other laboratories (6, 65) do not support this hypothesis.
5.1.1 The Effect of Lipid Composition

Our data indicate that the interaction of TAT-PTD with the lipid bilayer involves two phases. With the pure PC SUV, 24% of the binding occurred with the apparent dissociation constant $K_{d1} = 2.6 \pm 0.6 \mu M$, while the remaining 76% of the interaction had the apparent dissociation constant $K_{d2} = 610 \pm 150 \mu M$. Increasing the density of negative charge in the membrane caused a significant decrease in the value of the apparent dissociation constant $K_{d2}$. To maintain the physiologic relevance, the negative charge on the lipid was never increased above 30% in our experiments. Within these limits, the strength of interaction increased with the density of negative charge on the surface of the lipid vesicles (Figure 3.2). The interaction is unaffected by changing the chemical nature of the negative charge, such as replacing PG with PS (Figure 5.1), but it is attenuated in the presence of high salt (Figure 3.5). Fluorescence anisotropy of tryptophan, another measure of binding, also showed a greater increase with increasing negative charge in the membrane (Figure 3.8). Our data thus confirm that electrostatic forces between the basic peptide TAT-PTD and negative charges on phospholipids play a major role in the membrane binding of the peptide. However, non-electrostatic forces cannot be neglected in binding of TAT-PTD to the membrane. They contribute about 24% to the extent of binding, as revealed by the presence of a phase that was unaffected by high salt and also appeared in binding to membranes composed of pure neutral lipids. Our fluorescence-derived binding isotherms compare very well with those obtained by Ziegler et al. (112) by isothermal titration calorimetry. The latter authors explained their data in terms of Gouy-Chapman theory and concluded that electrostatics account for 77% of the binding, which we confirm herein. The value of the apparent dissociation constant for this process,
610 μM, also agrees well with their overall binding constant $K_{app}$, ranging between $10^3$ and $10^4$ M$^{-1}$.

![Graph](image)

**FIGURE 5.1:** Comparison of the interaction of TAT-PTD with (■) 25% PG - SUV, and (▲) 25% PS – SUV.

The peak of the tryptophan fluorescence emission was at 360 nm and its position did not change upon the addition of lipid, regardless of the presence or absence of negative charge in the bilayer. This indicates that the N-terminal tryptophan remains in the polar aqueous environment even upon binding to the membrane, which was confirmed by the ability of acrylamide to completely quench tryptophan fluorescence of the membrane-bound peptide (Figure 3.15).

Interaction with the DMPC/DMPG (3:1) membrane caused significantly greater changes in fluorescence of TAT-PTD when the membrane was in the liquid-crystal state, above its transition temperature (Figure 3.9). This indicates that the selective aggregation of negatively charged lipids by the cationic TAT-PTD peptide, implicitly assumed in the work of Ziegler et al. (112) and others, is required for strong binding to the membrane. We tested this directly in experiments with pyrene-labeled PG, which exhibits excimer...
fluorescence when the fluorophores are sufficiently close to each other. A greater than 100% increase in the excimer-monomer ratio was observed in the DMPC/DMPG membrane in the liquid-crystalline state, as compared with increase in the gel phase. These experiments demonstrate that, apart from the presence of negative charge, membrane fluidity is another important determinant for efficient binding of TAT-PTD to the membrane. On the other hand, these results suggest that binding of TAT-PTD may alter the physico-chemical properties of the membrane, namely, it may cause phase separation of lipids into distinct microdomains. A change in membrane properties upon binding of another cationic peptide was recently suggested by Hitz et al. (116). A cartoon in Figure 5.2 illustrates our observations on the predominantly electrostatic binding and on the peptide-induced lateral segregation of acidic lipids in the membrane.

5.1.2 Membrane Potential Studies

Membrane Potential Studies were carried out in order to see, first, if TAT-PTD binding perturbs the membrane to a significant extent so that ions can leak through, and second, if the membrane potential (negative inside), inherently seen in the living cells, can assist cationic peptides in crossing the high-energy barrier during their internalization. Our data show no dissipation of membrane potential induced by TAT-PTD, whether it interacted with 100% egg-PC LUV (Figure 3.12) or with 3:1 egg-PC/PG LUV (Figure 3.11) with similar membrane potential. This contradicts the conclusion of Henriques et al. (117) who even called the presence of membrane potential the sine-qua-non condition for transmembrane translocation of the synthetic CPP pep-1. The discord with our results may be due to, in part, by their experiments being carried out with pep-
1/β-galactosidase complexes rather than the naked peptide; the nature of their test for translocation, which was based on protection of β-galactosidase from digestion by trypsin applied from the cis side; and, most obviously, by the different chemical nature of the peptide: pep-1 carries smaller positive charge at neutral pH, but is much more hydrophobic than TAT PTD. Ziegler et al. (112) reported that TAT-PTD did not induce leakage of entrapped calcein (molecular mass of 623 Da) from lipid vesicles, and we extend that conclusion to the case of small ions; even temporary opening of passageways for ions would manifest in membrane potential dissipation. We also tested if the presence of membrane potential affects binding of TAT-PTD to the membrane. Limited stability of membrane potential in vesicles precludes determination of apparent dissociation constant from titration experiments, hence we only monitored fluorescence of the bound peptide before and after establishing the membrane potential. We observed no change in intensity or wavelength shift, which indicates that membrane potential does not appreciably influence the manner in which TAT-PTD binds to the membrane.

5.1.3 Insertion of TAT-PTD into the Membrane

From the measured efficiency of FRET we calculated the distance between the N-terminal tryptophan on the bound TAT-PTD and the membrane probe TMA-DPH as 3.4 nm. The charged head group of TMA-DPH anchors the probe at the membrane/water interface, oriented in parallel with the phospholipid acyl chains. Considering that there is only one neutral amino acid (glycine) between the terminal tryptophan and the first positively charged amino-acid residue (arginine), the large distance is surprising. A possible explanation might be that the positively charged TMA-DPH was repelled by the
positive charges on the peptide (the nearby N terminus and the cluster of basic amino-acid residues) and, consequently, the measured distance is not the expected shortest distance between the two fluorophores along the normal to the membrane (i.e., with the probe located directly under the peptide’s tryptophan). There was no observable FRET between the tryptophan and the uncharged hydrophobic probe DPH, which partitions predominantly in the hydrophobic core of the lipid bilayer. This indicates that the distance from the tryptophan to DPH is larger than that to TMA-DPH. These data, in conjunction with the quenching data, allow us to conclude that the N-terminus of TAT-PTD does not insert into the lipid bilayer. The value of fluorescence anisotropy of neither TMA-DPH nor DPH was affected by TAT-PTD binding, which indicates that TAT PTD does not have any effect on the fluidity of the lipid bilayer. Hitz et al. (116) very recently reported rigidification of the PC/PG bilayer by oligoarginines, based on the peptide-induced decrease in the excimer-to-monomer ratio of pyrene PG. It is hard to explain the peptide-induced increase in pyrene monomer fluorescence observed in that work. We saw just the opposite: upon interaction with the basic peptide, the monomer fluorescence decreased because the energy was rather emitted by excimers, whose fluorescence increased concomitantly—due to sequestration of the negatively charged pyrene PG by the basic peptide. We prefer this interpretation to the alternative of an increase in membrane fluidity because the latter was ruled out by our DPH fluorescence anisotropy data. Several factors may contribute to the difference between our conclusion and that of Hitz et al., including their use of LUV vs. our SUV and, perhaps even more significantly, the very high concentration of PG (70%) in their membranes: the much more abundant unlabeled PG might have excluded the pyrene-PG from significant binding and
sequestration that would manifest itself as an increase in the excimer-to-monomer fluorescence ratio. At any rate, the lack of the peptide effect on overall membrane fluidity, which we observed, suggests that no portion of TAT-PTD inserts into the bilayer. If it did, one would expect an increase in the anisotropy values, particularly that of TMA-DPH, as a consequence of an increase in surface pressure and a decrease in free volume in the membrane after peptide insertion. It is worth noting that Ziegler et al. (112) also reported that TAT PTD did not insert into a lipid monolayer at surface pressures that are close to physiologic one.

5.1.4 Effect of glycosaminoglycans

As we discussed earlier, both endocytotic and non-endocytotic mechanisms were proposed (21), with or without a simultaneous glycosaminoglycan-assisted internalization (118). More recently the reported internalization of whole proteins fused to CPPs has been attributed to the fixation artifacts (60), which caused a serious concern about the interpretation of the data from fluorescence microscopy studies and other indirect methods. The mechanics of TAT-PTD interaction with the naked lipid membrane (with varying surface negative charge), using fluorescence spectroscopic techniques was discussed above. The direct studies on TAT-PTD (42) and antennapedia homeodomain (119) with naked lipid bilayer from other laboratories also supported our argument that no evidence of peptide translocation is apparent in our model system. In the present study we focus on the interaction of TAT-PTD with lipid vesicles (with 25% PG) in the presence of saturated concentration of free glycosaminoglycans in the external environment under in vitro conditions.
In living tissues glycosaminoglycans are present in the basement membrane, extracellular matrix, and on the cell surface plasma membrane. Our study, although in vitro, can still be considered of biological significance because the glycosaminoglycans freely floating in the buffer external to the lipid vesicle SUV can be considered biological equivalent of the same in the extracellular matrix. Esko et al. (120) gave a detailed description of the ligand binding sites on heparan sulfate. Belting (118) (and references therein) state that the predominant forces in the CPP-heparan sulfate interaction are electrostatic. We confirmed this in the in vitro interaction of TAT-PTD with the three glycosaminoglycans, namely: heparan sulfate, dermatan sulfate, and low molecular weight heparin. Furthermore, we observed complete dissociation of TAT-PTD bound to glycosaminoglycans after addition of 2 M NaCl (Figure 3.19); at the same time, understandably no interaction was detected in the presence of high salt. The low dissociation constant (meaning better binding affinity) of TAT-PTD to low molecular weight heparin (Table 1) can be explained by higher concentration of sulfate per polymeric chain of low molecular weight heparin. However, although the sulfate concentration is the same in both dermatan sulfate and heparan sulfate, better binding to dermatan sulfate over the other was probably due to the higher molecular size of dermatan sulfate.

Several studies have stated the importance of heparan sulfate and other glycosaminoglycans in the internalization of TAT-PTD (63, 64, 104). However, Silhol et al. (65) have shown internalization of TAT-PTD into the cells lacking surface heparan sulfate (either due to enzymatic heparinase or mutation). In our experiments we have not seen glycosamonoglycans assisting the peptide in penetrating into the model lipid bilayer,
evidenced but lack of blue shift. On the contrary, these polyanions significantly inhibit
the interaction of TAT-PTD with SUV (Figure 3.20). It can be noticed that to maintain
biological relevance the concentration of glycosaminoglycans (0.1 mM) was relatively
high, as live cell cultures have around $10^5$ to $10^6$ molecules per cell (104). The
differences in the binding constants between the three glycosaminoglycans can be
explained by the differences in the sulfate concentration, molecular weight, and
excessively high concentrations of these molecules used in our experiments. The detailed
analysis on the difference between these binding constants is not the central idea and has
been considered a bit out of scope of our present study. However, it can be easily noticed
that the apparent dissociation constant $K_{d2}$, attributed to the electrostatic nature of the
interaction is an order of magnitude higher (meaning the binding is weak) in the presence
of glycosaminoglycans as compared against their absence (Table 1). The strength of
interaction of TAT-PTD with 25% PG SUV in the presence of glycosaminoglycans is
just within a comparable range from the interaction in the presence of high salt
concentration. The much weaker interaction in the presence of glycosaminoglycans as
against high salt can be explained by the polyanionic macromolecular nature of the
former over the small univalent nature of the later. All in all, we noticed an inhibition in
the interaction of TAT-PTD with model membrane in the presence of
glycosaminoglycans. These data should not be very surprising, as electrostatic forces
needed in the initial interaction of TAT-PTD with lipid membrane (13, 42), when
shielded by polyanions like glycosaminoglycans, should logically inhibit the interaction.
Richard et al. have also raised major questions on the inherent penetrating capacity of
TAT-PTD through hydrophobic core of the bilayer both in the presence (6) and absence

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of glycosaminoglycans. Our data are in accord with them and several others who suggested against a positive role of glycosaminoglycans in TAT-PTD insertion into the membrane.

![Diagram of Gel State (7°C) and Liquid-Crystal State (37°C)]

FIGURE 5.2: Proposed model of the interaction of TAT-PTD with model membrane.

5.2 Dendrimers

The promising claim of PAMAM dendrimers as potential intracellular drug delivery agents \((74, 75)\) has opened a lot of attention to this field. When the basic thermodynamics were considered the results remain a bit elusive, as it is hard to imagine a cationic dendrimers to pass through the hydrophobic core of the lipid bilayer without disturbing the cell membrane integrity, as was the case with some CPPs \((6, 13)\).

However, the potential cytotoxic effect inherent in the higher generation dendrimers, noticed by a >50% release of calcein from SUV \((78, 93)\), emphasizes the need for further \textit{in vitro} research before their biological/medical application. Some studies have shown that the higher generation dendrimers were more disruptive on LUVs \((93, 96)\). This can implicitly mean higher toxic effect of higher generation dendrimers. However the lower
generation dendrimers were assumed to be generally safe on Caco-2 monolayer and other cell lines\( ^{(85)} \). Due to this reason, lower generation can be more promising as potential therapeutic agents and nonviral transfection agents \( ^{(121-123)} \). The use of the model system has allowed us to characterize the membrane interaction of G1 and G4 dendrimers as a function of physicochemical properties of the lipid bilayer.

### 5.2.1 The Effect of Lipid Composition

We observed an increased affinity of the dendrimers to SUV with increase in the negative charge on SUV. Values of apparent dissociation constant \( (K_d) \) for G1 and G4 PAMAM dendrimers were found to be 30 ± 16 μM and 11 ± 3 μM, and 16 ± 7 μM and 5 ± 1 μM, for 0% and 25% negatively charged SUV, respectively. Further, complete dissociation of dendrimers with SUV, was noticed at high external salt concentration (Figure 4.2A, 4.2B). This confirms the pure electrostatic nature of the interaction, which implies that the interactions are predominantly between the surface groups of the dendrimers and the lipid bilayer. We did not find any involvement of non-electrostatic interactions (e.g., hydrophobic), which confirms observations from other labs \( ^{(78, 96)} \). Wang et al. \( ^{(124)} \), using isothermal titration calorimetry and dynamic light scattering, showed pH dependent changes in binding of the anionic detergent SDS to G3 PAMAM dendrimers, which is consistent with the electrostatic nature of the interaction. Due to strong light scattering from SUV in acidic environment, pH-induced changes in fluorescence anisotropy of labeled dendrimers could not be studied.
5.2.2 The Membrane Disruption studies

The cytotoxic effect of the low generation dendrimers was measured in vitro using calcein leakage from SUV. We observed a very minimal but significant membrane disruption by dendrimers. When 25% PG SUV interacted with G4 dendrimer (Figure 4.3), we saw a release as small as 7%, which is in accordance with the results of Zhang et al. (93). The release was found to be similar (with in the sensitivity of the instrument and experimental conditions) when the same experiment was performed with neutral SUV (5%). The release was much lesser with G1 than G4, and found to be 3% with anionic SUV and 0% with neutral SUV. The other significant finding in this study was that we did not find any dose dependence in the dendrimer-induced calcein release (Figure 4.4). We find this observation significant, as cytotoxic effect of most of the pharmacological drugs in general is assessed by their ability to release calcein in a dose-dependent manner (97). Based on our data, the actual cytotoxic effect of dendrimers remains controversial: a small effect seems to be there, but the lack of dose dependence indicates that the observed leakage may be due to an artifact. On the other hand, the fact that the release is dendrimer-generation-dependent cannot be ignored (93, 96). One possible way to reconcile this is that only the first dendrimers binding to the vesicle is able to rupture the membrane due to the necessity of large change in the shape of SUV. Those SUV that "survive" the initial shape change will not rupture when the subsequent dendrimer molecules appear—they may only aggregate the vesicles into SUV-dendrimer complexes, without additional leakage od calcein. The issue obviously needs further study. The rather low calcein release that we observed cannot account for the results of Mecke et al. (125, 126), who claim that dendrimers form holes in the lipid bilayer, as evidenced by atomic
force microscopy (AFM). Several factors may be contributing to this discrepancy. the first, they used higher-generation PAMAM dendrimers than us. Second, AFM usually works with planar bilayer or monolayer supported by a solid flat surface—it is possible that the strong electrostatic forces can overcome the cohesive hydrophobic forces in the bilayer and pull the lipid molecules out from the support, which would manifest as a “hole” in the AFM image. SUV membranes are flexible and can easily accommodate the shape of the bound dendrimer. To analyze the possible complex formation, we further studied this interaction by fluorescence quenching and distance measurements.

5.2.3 Vesicle aggregation

Zhang et al. (93) have shown with lipid mixing studies the apparent ability of higher generation dendrimers to aggregate lipid vesicles. We extend this to lower-generation dendrimers. We used fluorescence quenching of labeled dendrimers to obtain a better understanding interaction between the dendrimers and lipid vesicles. As shown in Figure 4.8 and 4.9, two populations of fluorophores with different quenching constants were found. It can be noticed that the population of fluorophores with lower Ksv (easily quenchable fluorophores) decreased with addition of lipid with a subsequent increase in the population of fluorophores with higher Ksv. Apparently, this is because of shielding of the fluorophores on dendrimers by lipid membranes. The shielding may be because the dendrimers penetrated through the lipid bilayer to the vesicle interior, where quencher has no access. But due to the apparent complete quenching both in the presence and absence of lipid vesicles and the complete dissociation induced by high salt, dendrimer internalization can be ruled out. This conclusion would be in accordance with data from
other authors who proved an absence of internalization using other fluorescence techniques [see ref (93)]. A more plausible explanation is that due to the presence of many positive charges on the dendrimer surface, the macromolecule is able to interact with several lipid vesicles and effectively aggregate them around itself, thereby hindering the access of the quencher to the fluorophore moiety. Dendrimer-induced vesicle aggregation can be experimentally verified by light scattering (turbidity measurements), which will be carried out in the near future.

Ottaviani et al. (127) have shown with dynamic light scattering, negatively stained TEM and EPR very little disruption of 100% neutral PC vesicles with G2 dendrimers. Our energy transfer data (Figures 4.6 and 4.7) show that the distance between the fluorophore to the lipid surface is shorter with larger dendrimers. If dendrimers cannot aggregate lipid vesicles and stay on the surface of the vesicles the distance should have been longer with higher-generation dendrimers. This leads us to conclude that G4 dendrimers have higher lipid-aggregation capability than G1 dendrimers.

5.2.4 Membrane Fluidity

Lateral phase separation in anionic membranes, induced by polycationic macromolecules, have been studied by measuring the pyrene excimer/monomer ratio of pyrene-labeled lipids (13, 128-130). In our data (Figure 4.4 and inset therein), we found that dendrimer-induced aggregation of PG in anionic membranes increased with increasing surface charge density. Furthermore, studies of phase dependence (Figure 4.5) have shown that the dendrimers bind stronger to membranes in liquid-crystal phase than
in gel phase. These results are in accordance with observations of Khopade et al. (131) on G4 dendrimer using differential scanning calorimetry.

Based on our results, we conclude that the membrane physicochemical properties, in particular fluidity, is a prerequisite for the efficient dendrimer-membrane interaction. Interestingly, Gardikis et al (2006) (132) using DSC and Raman spectroscopy studied the opposite effect, namely the effect of dendrimer on membrane properties, and found a concentration-dependent dendrimer-induced increase in membrane fluidity.

5.2.5 Membrane Penetration

The effect of lipid membrane packing and surface pressure on the interaction of surface active macromolecules to membranes was studied using Langmuir trough (133, 134). An increase in surface pressure at initial pressure < 20 mN/m suggests the ability of dendrimers to insert into the membrane at lower surface pressure (Figure 4.11). Such low surface pressure can be seen only in cells with rapid mitotic division like cancer cells and epithelial surface lining with rapid turnover (75, 88). The findings from this set of experiments could prove helpful in designing drug-delivery macromolecules in cancer treatment. Nevertheless, negligible changes in surface pressure at physiologic pressure (29-33 mN/m) support the notion that dendrimers are not internalized by lipid vesicles. The negligible increase in surface pressure might be attributed to the dendrimer-induced membrane curvature as suggested by Zhang et al (93). Although, the dendrimer induced adoption of inverse hexagonal phase by the lipid membrane [as proposed in ref (93)] is

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not addressed in our experiments with monolayers, we cannot rule out its possibility in model bilayers. It also can be a factor in dendrimer-induced lipid vesicle aggregation.

5.3 Overall conclusions on cell penetrating systems

Our data confirm that electrostatic forces play a major role in the initial interaction of TAT-PTD with lipid bilayer (6, 13, 42, 112). In physiologic range, the strength of interaction increased with the density of negative charge in the membrane. However, there is a second phase in the binding, which is attributed to non-electrostatic forces, such as hydrophobic or van der Waals forces. It is possible that a conformational change in the peptide during the interaction makes a thermodynamic contribution to this step. The peptide binding does not compromise the integrity of the lipid bilayer and does not perturb the latter in any significant degree, so that molecules or ions do not leak from vesicles. An important new result of ours is that the efficient binding of TAT-PTD requires fluid membrane with lipids in the liquid-crystal state. We explain this requirement in terms of the peptide-induced selective segregation of the negatively charged lipids, which is necessary to neutralize the positive charges on the peptide. However, this does not imply formation of separate large macrodomains of negatively charged lipids within the membrane. Although the actual internalization of the cationic protein transduction domain was not directly addressed in the present experiments, our data support the cautious conclusions of Ziegler et al. (112) and Richard et al. (6), namely, that no evidence of peptide translocation is apparent in our model system.

There are several controversial and contradictory reports on the role of glycosaminoglycans in the TAT-PTD/membrane interaction. Based on our data, we question the potential role of glycosaminioglycans in the translocation of CPPs through
the cell membrane. Our results not only support the conclusions of Silhol et al. (65) and Richard et al. (6), namely, that glycosaminoglycans do not play a major role in the penetration of TAT-PTD into cells, but extend it further by stating that at least *in vitro* they may even inhibit the interaction of TAT-PTD with model membrane.

There is still a lack of consensus on the mechanism of the dendrimer-membrane interaction, but, based on our data, we support the conclusions by other authors (93) that lower-generation dendrimers disrupt the lipid membrane very little, if at all. Our contribution to the present knowledge in the field is that membrane fluidity is a prerequisite for efficient dendrimer-membrane interaction. Efficient penetration through the lipid monolayer at lower surface pressures warrants further research, mainly focusing on potential application of the lower-generation dendrimers as potential anti-cancer therapeutic agents.

Based on the comparison of our data from TAT-PTD and dendrimers, we conclude that these two kinds of CPM have a different mechanism of action, at least *in vitro*. TAT-PTD, owing to its linear nature, localizes on the surface of the lipid bilayer and selectively aggregates the anionic lipids, but does not penetrate through the lipid bilayer in an appreciable degree. In contrast, dendrimers, with their globular nature, are able to aggregate lipid vesicles, possess mild membrane disruption property (which depends on the dendrimer size) and are able to insert into a lipid monolayer at lower surface pressures (while having no effect at physiological surface pressure). These results indicate different mechanism of interaction of linear and compact CPMs.
CHAPTER 6
Future Perspectives

6.1 Study with other cell penetrating peptides

In our studies the TAT-PTD was used as the model cell-penetrating peptide. However, as mentioned in Chapter 1, differences were noticed among various different CPPs identified to-date. Similar studies on other CPPs, might provide a more general platform for mechanism of CPPs. For example, there is no comprehensive study that would systematically investigate the effect of the amount, density, or distribution of positive charge on CPP. Also, it would be interesting to study peptides with added hydrophobic stretch of amino acids at various positions within the sequence.

In our present research, we used TAT-PTD labeled at the N-terminus end. Our data suggests that the tryptophan on the N-terminus end is approximately 3.4 nm above the membrane surface or, more precisely, above the level of the hydrophobic core of the membrane. (That is where the fluorophore TMA-DPH is anchored.) Better insight into the mechanism of action of these peptides can be got by using above proposed peptides (varying basicity and hydrophobic stretch addition), labeled at various sites: for example, in the middle of the cationic stretch or in the middle of the hydrophobic stretch, etc. The map of distances measured by FRET can give a better understanding of the architectural arrangement of CPPs on the surface of the lipid membrane and can be used to prove or disprove the hypothesized models like α-helix formation by CPPs on cell surface (45-47). Fluorescence spectroscopy would be a good technique to study this, because due to the
highly flexible nature of CPPs X-ray crystallography, a direct method for determining the structure of macromolecules, cannot be used.

6.2 Study with larger synthetic cationic peptides

In our present study, the TAT-PTD, which we used is a Tat protein fragment that has just 8 basic amino acids. The experiments can be repeated with a synthetic peptide with a greater stretch of basic amino acids like up to 32 or 64. This study would provide a comparison with smaller peptide sequences. The longer stretch, due to its enhanced surface interactions, may prove to be more membrane disruptive and it might be used in production of peptide based anti-bacterials and in other drug research. This insight will help the scientific arena in identifying the critical stretch needed for the optimum action of CPPs. Perhaps a new hypothesis can evolve based on the new understanding of the peptide-lipid interaction biophysics. In our studies, we have seen the interaction of these basic polymers increase with increasing negative surface density on the membrane. The negatively charged lipids in normal human cells range from 23 to 33% (predominantly in the inner leaflet of the bilayer), whereas in Gram-negative bacteria they make as much as 70% of all lipid. This might find a practical application in peptide antimicrobials selective against specific bacterial types and strains.

6.3 Insertion study with higher generation compact polymers

In our research on dendrimers, we used lower-generation dendrimers (G1 and G4). In future, we would like to extend our research to all generations of dendrimers from G1 to G8. It has been shown by Zhang et al. (93), that higher generation dendrimers have
membrane lytic property. In their leakage studies using higher generation dendrimers (G5-G7), the authors observed leakage as high as 70-80%. This study along with our research warrants a comparative study on all generations of dendrimers. Furthermore, in our surface pressure experiments, we observed greater increase in the surface pressure at lower initial surface pressure. This might be indicative of the penetration ability of lower generation dendrimers. An in situ study on the rapidly dividing cancer cell lines, using fluorescence microscopy or other microscopy technique, can help to prove or disprove this hypothesis. Comparative study of all different generations of dendrimers on the interaction with the lipid monolayer can also give a better insight into the mechanism of action.

6.4 Comparison of synthetic compact polymers vs. synthetic linear polymers

Dendrimers, due to their compact nature, have a greater surface charge density than linear molecules. Comparative studies with synthetic linear polyamines, can give us a better understanding of the differences in mechanism of action between linear and compact cationic polymers. Maybe the linear polymers are less membrane disruptive that higher-generation dendrimers and can find better physiologic applications than the latter. Further, preferential penetration of lower-generation dendrimers at lower surface pressure might warrant research on their specific capability to translocate chemotherapeutic agents attached to them in cancer treatment.
We believe that the presented and proposed work on cell-penetrating systems will open up new venues of research with potential applications in the treatment of various diseases and disorders affecting mankind.
References


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