

**Protein Targeting to Metal-Chelating Lipid Monolayers:
Toward New Materials Inspired by Nature**

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Abstract

Biological membranes are functional structures responsible for controlling numerous cellular functions. Many membrane functions (for example, transmembrane signaling and endocytosis) are the direct result of the dynamic nature of the membrane. The capacity to mimic biomembranes can lead to applications such as the production of biosensors and targeted drug delivery systems. In this work, a synthetic membrane-based recognition system capable of reproducing important features of biological targeting and assembly was developed. This system may open new routes to controlling molecular architecture in materials and devices, as well as produce new, functional materials.

Proteins are targeted to membranes through the affinity of surface histidine side chains for transition metal ions immobilized in the lipid headgroups. A set of metal-chelating lipids for this purpose was previously synthesized in our laboratory. Through measurement of the lipid surface pressure-molecular area (π -A) isotherms, the capability of the iminodiacetate (IDA) headgroups to chelate Cu^{2+} was demonstrated; the apparent association constant for the IDA lipid-Cu complex is $1.3\text{-}1.8 \times 10^8 \text{ M}^{-1}$. In addition, to tailor the density of metal sites on the membrane, mixing of a pyrene-labeled IDA lipid with two phosphocholine lipids was investigated.

The binding of horse heart myoglobin to IDA-Cu lipid monolayers by naturally occurring histidines was observed through changes in the lipid π -A isotherms. In addition, targeting of a recombinant rat liver cytochrome b_5 , engineered to display a hexa-histidine affinity tag at the C-terminus, to IDA-Cu lipid monolayers was demonstrated. The protein binding was measured as a function of the type of IDA-Cu lipid composing the monolayer, the concentration of protein in the aqueous subphase, and the fraction of IDA-Cu lipids in the monolayer. The hexa-histidine tag was found to provide an apparent affinity for IDA-Cu lipid binding of at least $2 \times 10^7 \text{ M}^{-1}$. A Frumkin-type adsorption isotherm was developed to describe the binding of proteins to lipid monolayers.

The inherent two-dimensional order of biological membranes is also capable of promoting ordered growth of organic and inorganic biomaterials. Synthetic lipid monolayers can likewise induce growth of 2D protein crystals at the air-water interface. For the first time, growth of 2D protein crystals on metal-chelating lipid monolayers *via* coordination of naturally-occurring surface histidines is demonstrated. Two-dimensional crystals of the protein streptavidin, grown on IDA-Cu lipid monolayers, were observed using Brewster angle and fluorescence microscopies. The IDA-Cu-histidine interaction may provide a general route to the growth of 2D protein crystals for structure determination, as well as the production of functional materials based on the properties of the proteins, themselves.

Finally, many biological membrane functions are produced by ligand-induced assembly of membrane components (e.g. transmembrane signaling). Mimicking ligand-induced assembly and subsequent signaling can produce functional materials such as the IDA lipid-based metal ion sensor (Sasaki, *et al.* Angew. Chem. Int. Ed. Engl. **34**, 905-907, 1995). In order to probe similar ligand-induced reorganization of a pyrene-labeled IDA lipid in Langmuir monolayers, a UV-vis fluorescence microphotometer has been constructed. As a demonstration of its utility, the instrument was used to quantitate fluorescence from a pyrene IDA lipid monolayer and estimate a diffusion coefficient for the lipids. In addition, reorganization of the IDA lipids by binding of a multivalent ligand, poly(histidine), was observed *via* the pyrene lipid fluorescence.

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To Dad and Mom,

with all my love.

*The man who wants a garden fair,
Or small or very big,
With flowers growing here and there,
Must bend his back and dig.*

*The things are mighty few on earth
That wishes can attain.
Whate'er we want of any worth
We've got to work to gain.*

*It matters not what goal you seek
Its secret here reposes:
You've got to dig from week to week
To get Results or Roses.*

— Edgar Guest

Chapter 1

Introduction.

Nature as inspiration for new materials

The development of methods for controlling materials architecture at the molecular level is a current theme in materials science. The need for such materials is driven by the trend toward ever-decreasing size of materials and devices in virtually all areas of science and technology [1]. While technology has not yet reached the theoretical lower limit on size (according to the oft-quoted aphorism of Richard Feynman from his “Tiny Machines” lecture, “there’s plenty of room at the bottom” [2]), we are quickly approaching a limit set primarily by currently available materials. One solution to this problem is to develop new materials in which functions are performed on the level of single molecules, resulting in so-called molecular devices. Nature has spent literally billions of years developing elegant solutions to exactly this problem; the production of functions by single molecules in materials ordered on the molecular scale. In so doing, the living cell has become a master of materials science. For example, a typical microorganism is capable of efficient information storage (DNA comprises ca. 50 atoms per bit of data) with automatic error checking and damage repair; has built-in input, output and networking hardware; has a built-in power supply and is self-propelled, all within a package on the order of $1\ \mu\text{m}^3$.

The ability to mimic natural materials has led to a wide variety of new materials applications. For instance, cell surface receptors provide specific, high affinity binding of external ligands. Biosensors using a convenient electrical detection system were produced by combining lipid/protein mimics of biomembranes with appropriate electrodes [3]. Natural systems often use organic supramolecular scaffolds of proteins or lipids (or both) to induce growth of hierarchically-structured inorganic composite materials such as seashell and bone [4-6]. Using similar organic materials as templates, researchers have produced specifically oriented and shaped inorganic materials such as CaCO_3 [7-9], SiO_2 [10], metal sulfides (PbS , CdS , ZnS) [11-13] and Fe_3O_4 [14]. Finally, viruses and pathogenic bacteria have developed the ability to specifically infect particular host cells. In a similar

fashion, drug carrying particles such as liposomes and polymeric nanospheres have been functionalized with cell-specific receptors (most commonly antibodies), thereby delivering their therapeutic agent to specific cell types [15-17].

Membrane reorganization, targeting and assembly

The cellular membrane is remarkable even among nature's accomplishments. In addition to serving as a barrier to separate the interior of the cell from the surroundings, the membrane is a functional structure responsible for controlling all of the cell's interactions with its environment. Such diverse activities as hunting for food (chemotaxis) [18, 19] and communicating with other cells [20] are controlled by the membrane or membrane-bound components. The cell membrane consists mainly of a bilayer of lipids. Its functionality, however, is provided by the many different types of molecules embedded within or associated with the lipid matrix. The dynamic nature of the lipid matrix allows these components a high degree of mobility within the membrane. This lateral mobility makes it possible for membrane proteins to reorganize into the superstructures and multi-component assemblies responsible for membrane functions such as transmembrane signaling [21] and endocytosis [22]. In addition, the inherent two-dimensional order of the cell membrane is used in nature to control the growth of biominerals [4] or produce ordered, functional materials such as the purple membrane of the bacteria *Halobacterium halobium* [23].

Signal transduction by the family of G-proteins is one example of a function generated by external ligand-induced protein reorganization. The role of G-proteins in generation of a neural signal in the olfactory system is illustrated in Figure 1.1. Olfactory receptor proteins, specific for a wide variety of odorant molecules, are displayed on cilia in the nasal epithelium. Binding of a small molecule to the receptor protein induces a structural change which allows the protein to bind to a G-protein complex. Binding of the receptor leads to phosphorylation and activation of the G-protein α , which can

subsequently bind to and activate adenylate cyclase. Adenylate cyclase, in its turn, catalyzes the production of cyclic adenosine monophosphate (cAMP). The cAMP acts as an internal messenger which causes transmembrane ion channels to open, allowing monovalent cations to flow from outside the cell to inside. Finally, the change in potential across the membrane becomes the neural signal transmitted to the brain. It is the localization of each of these proteins within the membrane, and the ability of the proteins to reorganize and assemble with one another, which allows efficient transduction of the binding of the odorant molecule into a neural signal.

A second example of function generated by ligand-induced membrane protein assembly is apoptosis (programmed cell death) of T cells due to cross-linking of the Fas receptor protein by its trimeric ligand, FasL. Recently, Schreiber *et al.* have shown that dimerization of the Fas receptor by an external ligand is sufficient to induce cell death both *in vivo* and *in vitro* [24, 25]. Specifically, they have produced a genetically engineered, chimeric protein which can mimic the function of the native Fas receptor, consisting of the intracellular domain of Fas receptor, a membrane anchor and a specific ligand-binding domain known as FK1012-binding protein (FKBP) (c.f., Figure 1.2). The small membrane-permeable molecule FK1012 cross-links adjacent FKBP domains, thereby triggering dimerization of Fas and signaling apoptosis. Genetic abnormalities in the Fas receptor or FasL lead to massive T-cell proliferation and, ultimately, an autoimmune syndrome which has been ascribed to the absence of Fas-mediated apoptosis.

The mimicking of cell membranes in artificial systems can lead to many interesting applications. Just as receptor cross-linking *in vivo* can lead to triggering of the natural function of a protein, receptor cross-linking in synthetic systems can be used to generate useful functions. Using this approach, we have developed a membrane-based metal sensor, sensitive to parts-per-trillion Cu^{2+} concentrations in solution [26]. The metal-chelating lipid PSIDA (1-octadecyl-2-(9-(1-pyrene)nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid)) forms aggregates when placed in vesicles of

DSPC (distearoyl phosphatidylcholine). Because the fluorescent properties of pyrene depend strongly on its local concentration within the membrane (c.f. Chapter 5 and [27, 28]), the aggregates emit primarily as excimer species with a maximum fluorescence intensity at 470 nm. However, upon binding of metal ions by the iminodiacetate (IDA) headgroup, the PSIDA aggregates are essentially instantaneously dispersed into the DSPC gel matrix (Figure 1.3). The lower local pyrene concentration produces a decreased excimer fluorescence and an increased monomer fluorescence at 377 nm (Figure 1.4). Note, because the excimer emission is visible while the monomer is in the UV, the change in the emission spectrum is detectable to the naked eye at sufficiently high vesicle concentration. The effect is responsive to a wide range of divalent cations, and the sensitivity largely parallels the known affinities of the various ions for IDA in solution. This lipid reorganization in response to metal chelation amounts to a ligand-induced disassembly, as opposed to assembly, of the membrane components, but the concept of receptor reorganization producing a function remains the same.

Two-dimensional protein crystallization

While being dynamic structures, biomembranes are also highly ordered in two dimensions. Living cells are capable of utilizing the inherent order of cellular membranes as a template for directing the growth of organized organic and inorganic materials. A striking example is the ability of lipid membranes to direct the growth of various biominerals *in vivo* [6, 29]. In unicellular diatoms, the ultimate shape of intracellular biominerals such as amorphous silica is determined by the structure of membrane-bound compartments [30]. Another example is the surface layer proteins (S-layers) which form the outermost layer of many bacteria. S-layers are two-dimensional (2D) protein crystals on the outer cell membrane which act *in vivo* as structure maintaining elements, protective coats, molecular sieves, and recognition elements [31, 32]. However, S-layers have also been employed synthetically as new materials for the construction of molecular filters with

steep cut-off for exclusion [33], patterned immobilization and affinity matrices [34] and biosensors [35].

Interestingly, S-layer proteins reconstituted *in vitro* have been utilized as a photolithographic mask for production of nanometer-scale patterns [36, 37]. The S-layers were first deposited onto a smooth graphite surface. The proteins of this particular S-layer are organized in a hexagonal lattice, and the crystal contains “holes” between the proteins, also necessarily arranged in a hexagonal lattice. The surface of the protein crystal was evaporatively coated with a thin film of Ti, which subsequently oxidized in air to TiO_x . Fast-atom beam milling at normal incidence produced a pattern of holes in the underlying graphite governed by the pattern of holes in the S-layer crystal. The ability to pattern surfaces on the scale of a few nanometers is currently limited to serial methods such as scanning tunneling microscopy. Thus, the ability to produce nano-scale patterns over a large area opens a wide array of possibilities for production of devices and materials with electronic, chemical, or optical functions.

Again, it is possible to mimic membrane-controlled growth of ordered materials in synthetic systems. It has been known for many years that proteins can be crystallized in two dimensions on the surface of Langmuir monolayers [38, 39]. This most often entails using a fluid monolayer of lipids derivatized with an affinity ligand for the protein of interest (Figure 1.5). The protein is therefore highly enriched at the air-water interface (500-1000 mg/mL) [39]. The elevated protein concentration, in combination with the dynamic nature of the membrane, assists protein assembly into highly-ordered arrays and 2D protein crystals. In recent years, this technique has been successfully applied to a wide variety of proteins, including yeast RNA polymerases I [40] and II [41], *E. coli* DNA gyrase B subunit [42, 43], ribonucleotide reductase B1 [44] and IgG and IgE antibodies [38, 45, 46]. All crystallize in the presence of lipid monolayers containing specific affinity ligands.

Probably the best known example of 2D protein crystallization is that of streptavidin ($M_r \sim 60,000$) which binds the small molecule biotin very tightly ($K_a \sim 10^{15} \text{ M}^{-1}$ [47]). This protein has been shown to form 2D crystals on Langmuir monolayers of biotin-containing lipids [48, 49]. Streptavidin crystals are unique due to the distribution of biotin binding sites on the protein. Streptavidin is tetrameric, with four binding pockets, two on each side of the protein (c.f. Chapter 4). Thus, upon binding of two equivalents of a biotin-lipid at the air-water interface, two binding pockets remain available for further binding of biotin and are accessible to the aqueous subphase. These empty binding sites allow assembly of protein multilayers, using biotin-functionalized macromolecules, which may lead to functional materials [50, 51]. For instance, streptavidin crystals were utilized as a template for the attachment and organization of a layer of biotinylated antibody Fab fragments [52]. In fact, such a biotin-lipid/streptavidin/Fab triple layer was used to produce a prototype biosensor for the hormone human chorionic gonadotropin [51]. Finally, proteins such as biotinylated phycoerythrin [53] have been oriented on 2D streptavidin crystals for formation of functional materials.

Approach: metal-chelating lipid monolayers

We have seen that reorganization and assembly of membrane components is used to produce functions in natural systems, and mimicking of natural membranes has led to a variety of applications. However, most examples have relied on the use of natural membrane proteins which are reconstituted in artificial systems. We would like to be able to generalize membrane-based devices and materials by targeting a more diverse set of proteins, including those that are not naturally membrane-associated. This capability is key to our goal of developing simple and robust artificial membrane systems capable of reproducing some of the important features of biological membrane reorganization and protein assembly.

The first requirement for the production of functional, synthetic membrane systems is a mechanism for targeting proteins (and other biological ligands) to the membrane surface. A variety of non-specific interactions, utilizing both electrostatics [54, 55] and covalent bonds [56-59], has been developed for immobilization of proteins on surfaces. In contrast, targeting of proteins to lipid membranes has generally relied on interactions with specific lipid-bound receptors or affinity ligands. Some examples include haptens for antibodies [38, 60-62], antibodies for specific proteins [17, 63], inhibitors/effectors for enzymes [42, 44] and the heme prosthetic group for apomyoglobin [64]. This approach has two inherent disadvantages, however. First, it is necessary to design and synthesize a unique affinity lipid for each new protein of interest. Perhaps more importantly, no affinity ligands are available (or even known) for many proteins with interesting biological, optical, electronic or catalytic properties. Thus, a versatile, generally applicable affinity system for targeting and orienting proteins at membranes would be extremely useful.

In the current work, we have chosen to utilize the affinity of proteins for immobilized metal ions as a means of targeting proteins to membranes. Coordination to divalent transition metal ions, such as Cu^{2+} or Ni^{2+} , by the lone pair of electrons on either of the imidazole nitrogens of the histidyl side chain (Figure 1.6) provides an interaction that is relatively strong ($K_a \sim 10^{3.5} \text{ M}^{-1}$ for imidazole coordination to Cu^{2+} -iminodiacetate (IDA-Cu) [65, 66]), at least in comparison to a hydrogen bond or charge-charge interaction. Much higher affinities, comparable in fact to those of specific biological recognition systems, can be obtained by simultaneous coordination of metal ions by multiple functional groups on a macromolecular ligand (e.g. several histidines on the surface of a protein) [67, 68].

To utilize this approach for targeting proteins to lipid membranes, the metal ions must be immobilized on the membrane surface, yet accessible for protein binding. Several chelating lipids that can form ternary lipid-metal-protein complexes (Figure 1.7) have been

designed and studied in our laboratory; they are characterized in detail in Chapter 2. Each lipid utilizes the tridentate metal-chelating iminodiacetate (IDA) moiety as the hydrophilic lipid headgroup. IDA forms a charge-neutral complex with divalent transition metal ions. With Cu^{2+} , two strong coordination sites remain available for ligand (protein) binding. The IDA-Cu complex is suitable for targeting proteins through their native surface histidines as well as through His-tags and engineered metal chelating sites. IDA can also be used with Ni^{2+} for binding His-tagged proteins.

This interaction has been employed for several years in protein purification *via* immobilized metal ion affinity chromatography (IMAC) [69]. More recently, a hexahistidine “affinity tag,” genetically engineered at the N- or C-terminus of a protein, has been used to provide very high affinity for immobilized metals [70]. This approach has gained momentum in recent years, garnering widespread interest as a tool for efficient purification of recombinant proteins [71-74]. In addition, histidine tags have been used in diverse applications such as protein immobilization for atomic force microscopy [75] and production of catalytic surfaces [76], as well as metalload sorption by bacteria for bioremediation [77].

High affinity binding sites for metal coordination can also be genetically engineered onto the surface of a protein [78]. For instance, two histidine residues may chelate a single metal ion if positioned correctly on the protein surface. Configurations which support the correct binding geometry include His-X-His in a β -strand, His- X_2 -His in a reverse β -turn and His- X_3 -His in an α -helix (X represents any amino acid) [67]. A His- X_3 -His chelating site, for instance, can provide a binding constant for IDA-Cu as high as $2 \times 10^6 \text{ M}^{-1}$ [79]. Such chelating sites have been developed for protein separations [80, 81], as well as increased thermostability [82].

Metal coordination interactions offer several advantageous features for protein targeting to lipid membranes. Complexation is favored at neutral or slightly alkaline pH

and is reversible under mild conditions: lowering the pH or adding a chelating agent such as ethylenediaminetetraacetate (EDTA) disrupts the ternary complex formed by the metal, the lipid and the ligand (protein) (Figure 1.7). Unlike many specific and nonspecific biological interactions, metal coordination is not adversely affected by high salt concentrations. Furthermore, any of the large number of proteins with naturally occurring, surface-accessible histidines can be targeted to membranes by this approach. Because histidine is primarily responsible for protein interactions with the metal complexes at neutral pH, the site(s) on the protein surface responsible for binding are known. The locations of these sites can also be controlled by site-directed mutagenesis. Additional interactions with surface amine groups (lysine, N-terminus) can be exploited, if desired, at slightly alkaline pH. Finally, the hexa-histidine affinity tags and simple di-histidine chelating sites described above could be used to target protein to metal-chelating membranes with very high affinity and a specific orientation.

Project overview

This investigation will demonstrate the utility of the histidine-metal interaction for targeting of proteins to membrane surfaces. A set of metal-chelating lipids, employing the metal chelator IDA as the polar headgroup, has previously been synthesized. The metal binding properties of these lipids, formed in Langmuir monolayers at the air-buffer interface, will be studied in Chapter 2. Surface pressure-molecular area (π -A) isotherms verify the ability of each of the lipids to chelate CuCl_2 , either from the aqueous subphase or pre-loaded into the headgroup before spreading of the monolayer. Various possible applications require different membrane properties (fluidity, compressibility, phase transitions, etc.). Due to variations in the chemistry of the hydrophobic lipid tails, the set of metal-chelating lipids spans a wide range of such properties, as revealed by the π -A isotherms. In addition, a metal-chelating lipid containing a fluorescent reporter group (pyrene) affords an optical probe of the membrane architecture. In order to control the

density of functional groups (metal ions) on the monolayer surface, mixing of the fluorescent lipid with two neutral matrix lipids will be presented.

Binding of ligands to the lipids affects the packing and molecular arrangement of the monolayer. Thus, changes in the surface pressure-molecular area behavior of the lipids can be sensitive indicators of ligand binding. In fact, binding of proteins to the metal-chelating lipids will be investigated *via* this approach (Chapter 3). In addition, fluorescence microscopy allows direct observation of the protein at the monolayer interface. Targeting of proteins to IDA-Cu lipid monolayers is achieved by binding of both naturally occurring, surface-accessible histidine residues and a genetically engineered poly(histidine) affinity tag. The role of the IDA-Cu-His interaction in protein binding is confirmed. The protein binding is studied as a function of parameters such as lipid fluidity and metal ion density. A mathematical model is presented which describes the changes in monolayer area upon protein binding in terms of the protein concentration and the amount of IDA-Cu lipid in the monolayer. Negative cooperativity in protein binding is observed, and the magnitude of the cooperativity depends on properties of both the lipid (compressibility, molecular area) and protein (size).

In addition to merely binding protein, IDA lipid monolayers provide a surface for promoting two-dimensional protein crystallization. The protein streptavidin crystallizes on the highly fluid Langmuir monolayers of a di-unsaturated IDA lipid through coordination of naturally occurring histidine side chains (Chapter 4). While streptavidin has been known to crystallize in two dimensions on Langmuir monolayers of biotin lipids, this result is the first demonstration of 2D crystallization of any protein on metal-chelating lipid monolayers. The characteristic rectangular shape of streptavidin crystals grown on IDA-Cu lipid monolayers is quite different from the H-shape morphology of biotin lipid-bound crystals. The H-shape is thought to be the result of an asymmetry in inter-protein contacts due to binding of biotin in only two of streptavidin's four binding pockets [83]. The rectangular shape of the IDA-Cu-bound crystals is, therefore, most likely the result of the removal of

this asymmetry; all four biotin-binding pockets are free of biotin. This system is remarkable in that it represents a potentially general route to 2D protein crystallization which could greatly accelerate the investigation of protein crystals in both structural biology and materials applications.

Finally, we wish to investigate the reorganization of membrane components induced by binding of external ligands. The fluorescence of the pyrene-containing IDA-Cu lipid could be used as an optical probe of its membrane environment and, therefore, could signal its ligand-induced reorganization. In this light, the construction of a UV-vis fluorescence microphotometer for quantitation of fluorescence from Langmuir monolayers is described (Chapter 5). The instrument integrates a UV-adapted fluorescence microscope with photon counting electronics for simultaneous imaging and photometry of Langmuir monolayers. As an example of the power of the instrument, low-level pyrene fluorescence intensities are measured and used to estimate a diffusion coefficient of the lipids in the monolayer. In addition, aggregation and cross-linking of the pyrene IDA-Cu lipids induced by binding of multivalent poly(histidine) will be followed using the pyrene fluorescence properties. This result demonstrates the ability of the pyrene-containing lipid and the photometer to follow ligand-induced reorganization and assembly of membrane components.

Future research directions

This work should serve as a stepping stone, opening the door to a number of exciting possibilities for producing functional, protein/membrane-based materials. For instance, the metal-chelating lipids provide all the necessary properties for inducing 2D protein crystallization and may lead to a general route to formation of 2D crystals. In addition to their use in structure determination by electron crystallography, 2D crystals of electron transport or photoreceptor proteins, for instance, may display interesting properties for production of electronic or opto-electronic devices. In fact, an electrochemical photocell

constructed from a thin film of bacteriorhodopsin has been reported, which absorbs visible light producing a rectified photocurrent [84]. In general, organization of proteins into crystals would improve the absorption of optical signals in such devices by aligning the absorption dipole of the protein with the polarization of incoming light. The anisotropy of the protein components would also produce polarized emission or energy transfer, possibly improving coupling of the crystal to a secondary layer of a device. Furthermore, 2D protein crystals may have the fortuitous benefits of allowing more efficient packing of proteins on a surface and increasing the thermostability of the proteins [85].

Using the ability of protein to induce reorganization of the membrane components upon binding, it may be possible to produce surfaces capable of highly specific protein recognition based on the distribution of binding moieties on the protein surface. Binding of a protein displaying multiple histidine residues on a single face to a Langmuir monolayer containing a small fraction of IDA-Cu lipid will organize the functional lipids into a pattern mirroring the distribution of histidines. The pattern in the monolayer may then be fixed, for example by polymerization of diacetylenic lipids, resulting in a “recognition element” specific for the “template” protein (Figure 1.8). This technique, dubbed “molecular printing,” is the 2D equivalent of template polymerization in three-dimensional macroporous polymers (c.f. [86] for a recent review). Such patterned surfaces would have obvious applications in the production of biosensors. Note that virus particles, consisting of symmetrical aggregates of proteins, could also be used as templates, resulting in a sensor with utility in clinical diagnostics. Also, one should be aware that this approach is not restricted to the IDA-Cu-histidine interaction. Any number of lipid-protein interactions may be employed, and it may be possible to combine two or more interactions in a single experiment to produce highly specific recognition elements.

Finally, molecular printing may be combined with 2D protein crystallization to generate surfaces patterned on the molecular scale over very large areas. For instance, in the streptavidin crystallization on IDA-Cu lipid monolayers, two histidines per protein bind

to the metal-chelating lipids (see Chapter 4). As the proteins are organized in a regular “lattice,” the underlying IDA-Cu lipids are necessarily organized in the same lattice (Figure 1.8). Fixing the monolayer by polymerization (or some other means) would result in a pattern of IDA-Cu lipids governed by the lattice of the protein crystal extending in two dimensions over the area of the crystal (as much as 1 mm). In addition, the spacing of lipids in the pattern may be controlled by tailoring the distribution of histidines on the protein template through site-directed mutagenesis. Currently, no method for patterning surfaces on this scale exists, short of manually placing individual atoms *via* a probe tip microscope such as an atomic force microscope or scanning tunneling microscope. In contrast to these “serial” techniques, however, molecular printing is a “parallel” technique, simultaneously producing a number of patterns over large areas.

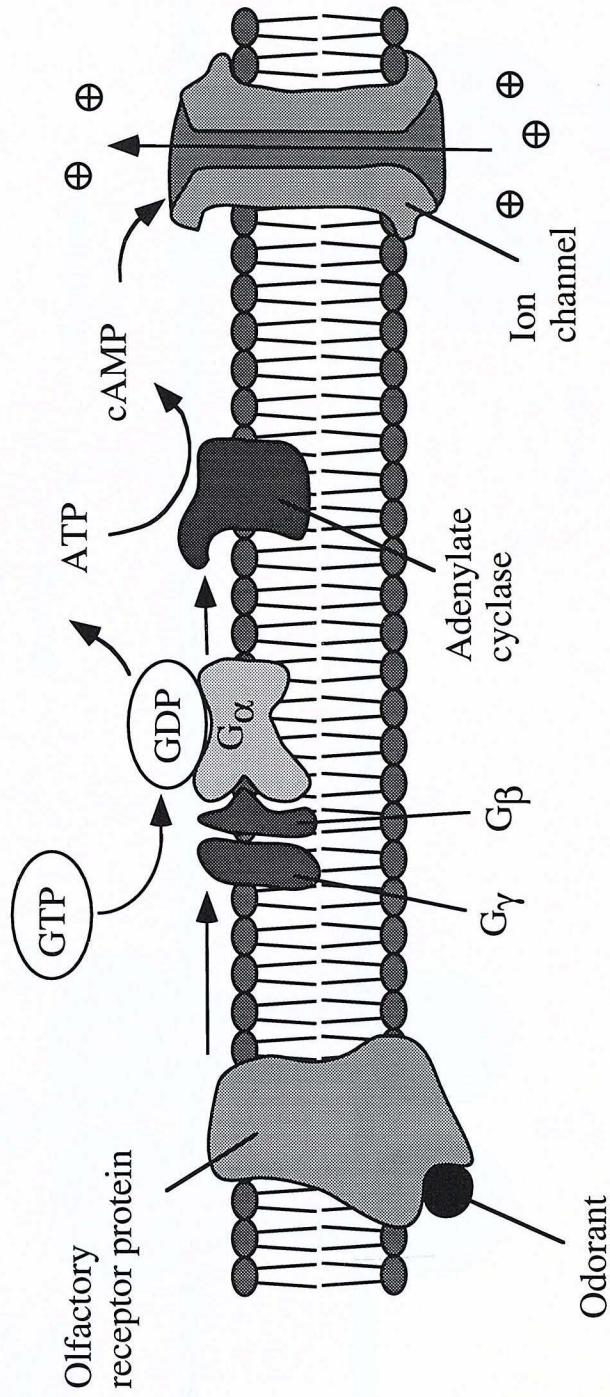


Figure 1.1. Binding of an odorant molecule to olfactory receptor proteins induces a cascade of protein-protein interactions, culminating in opening of ion channel proteins and transmission of a neural signal. G α , G-protein α ; G β , G-protein β ; G γ , G-protein γ ; GDP, guanosine diphosphate; GTP, guanosine triphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate.

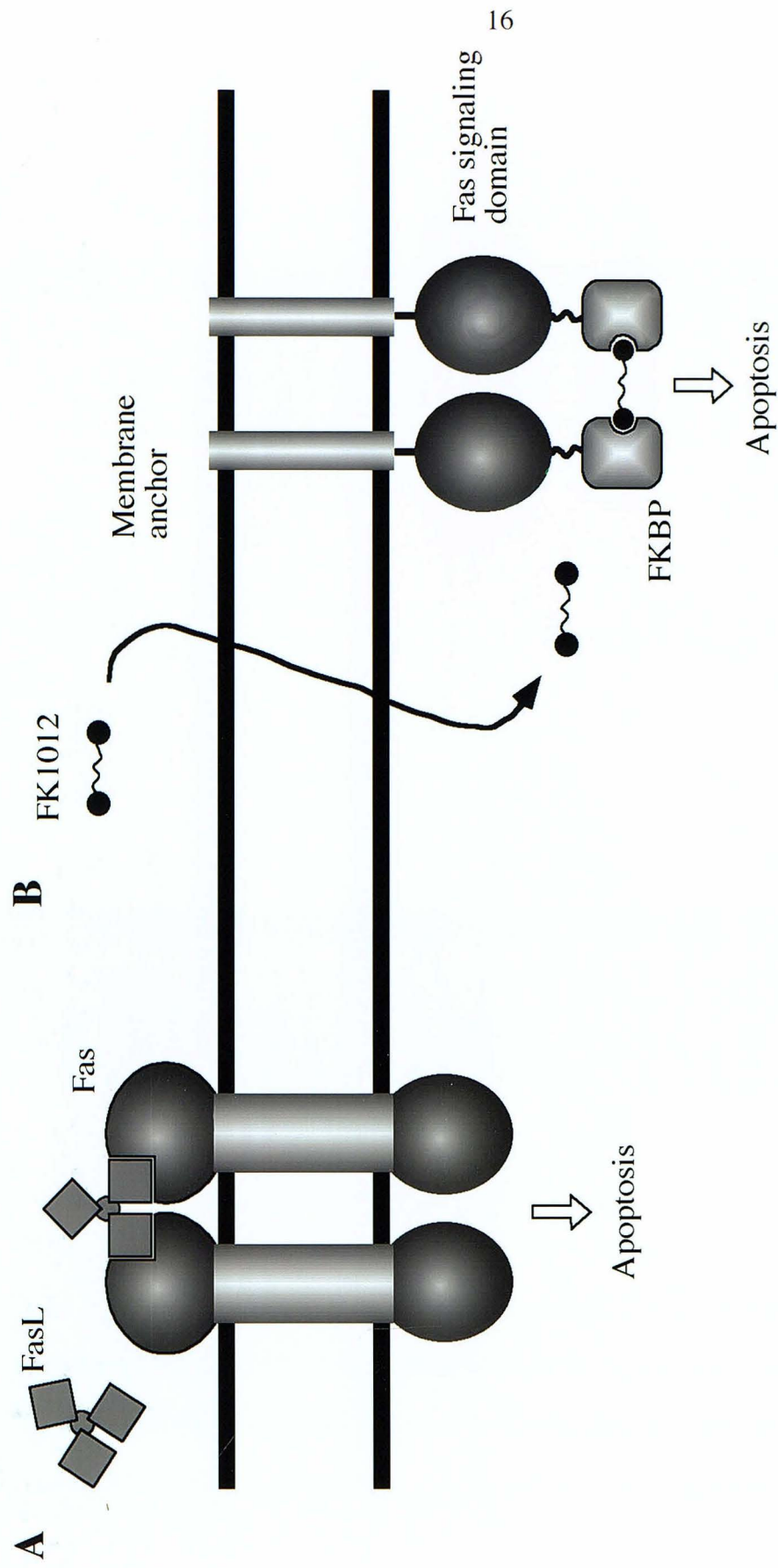


Figure 1.2. (A) Cross-linking of Fas by binding of its ligand, FasL, to the extracellular domain of the protein leads to signaling of apoptosis (programmed cell death). (B) Binding of FK1012 to its receptor, FKBP (FK1012-binding protein), cross-links the chimeric protein and dimerizes the Fas signaling domain, also signaling apoptosis.

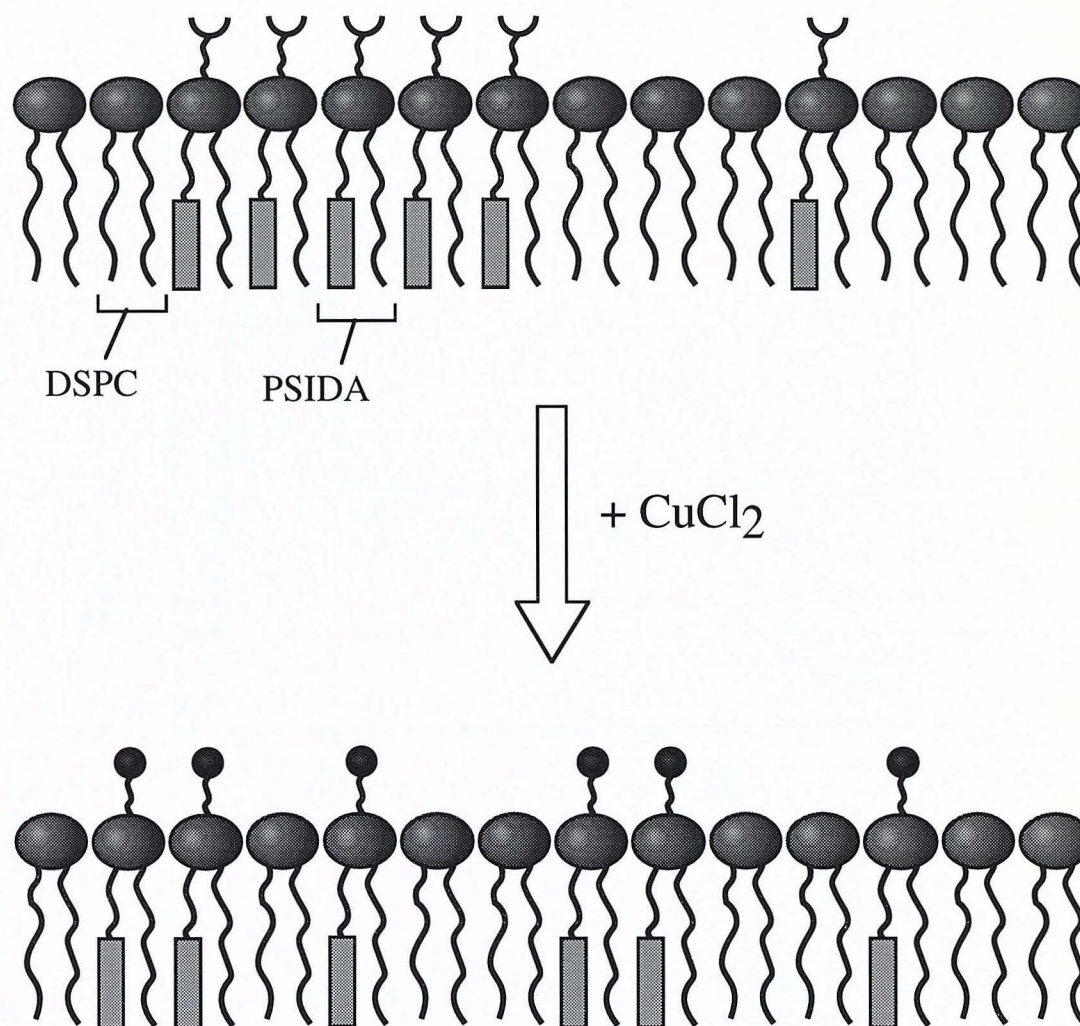


Figure 1.3. Schematic representation of metal-induced dispersion of PSIDA in a mixed vesicle of PSIDA/DSPC, as observed through fluorescence measurements. PSIDA is believed to assemble into pyrene-rich aggregates which are dispersed upon chelation of a divalent metal ion, such as Cu^{2+} , by the lipid headgroup.

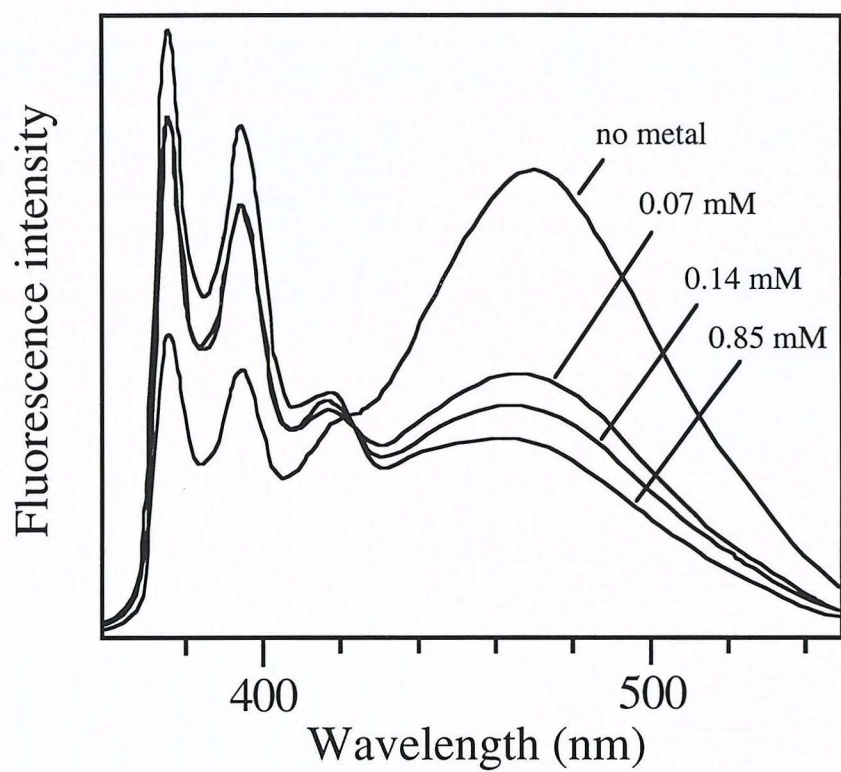


Figure 1.4. Fluorescence emission spectra of 5% PSIDA/95% SOPC vesicles in buffer (20 mM MOPS, 100 mM NaCl, pH 7.5) at 25 °C with increasing concentrations of MnCl_2 .

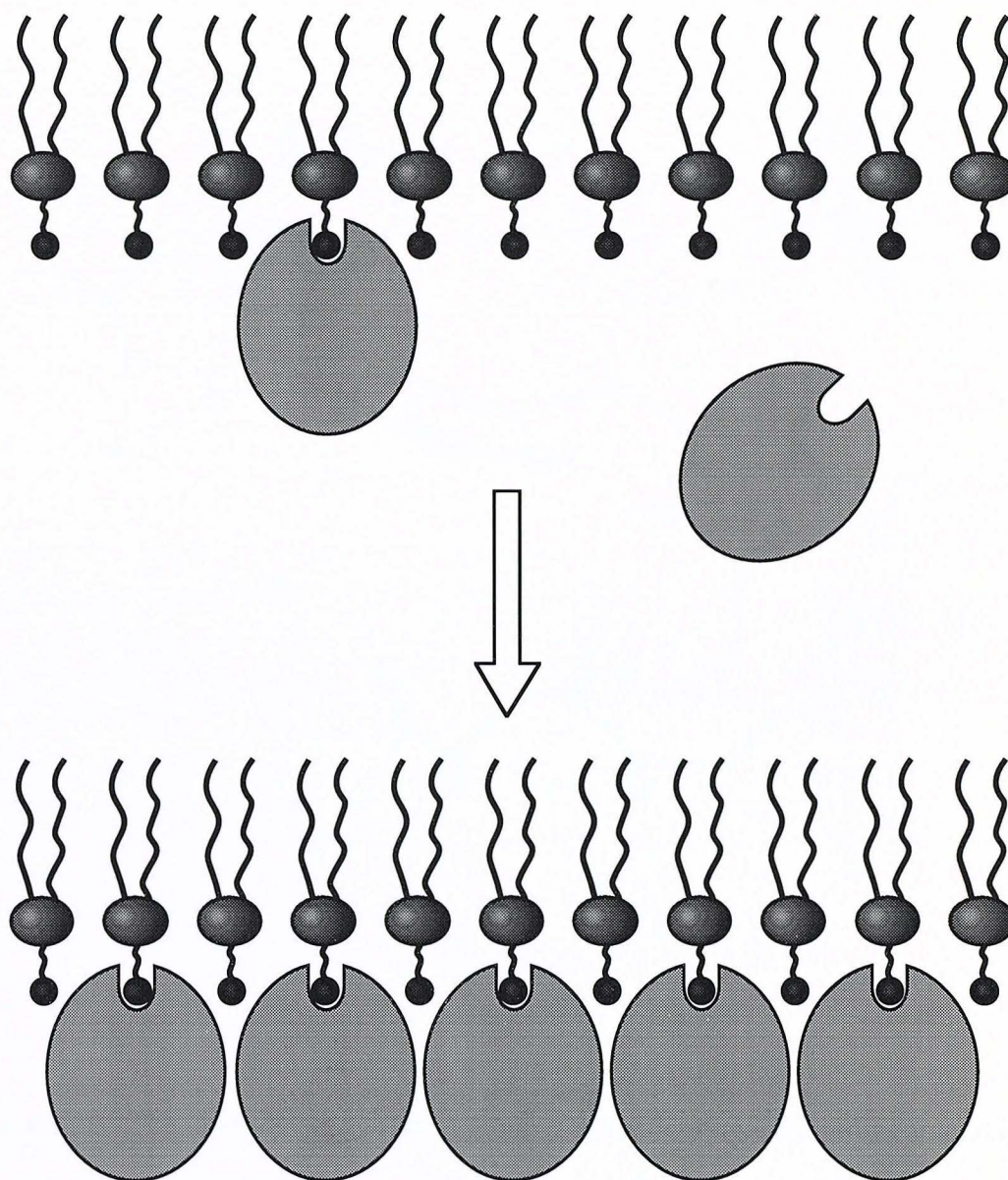


Figure 1.5. Schematic representation of 2D protein crystallization on Langmuir monolayers at the air-water interface. Specific ligands in the lipid headgroups target protein to the interface with a preferred orientation and in high concentration. The fluidity of the monolayer allows the bound proteins the mobility to sample inter-molecular contacts and ultimately organize themselves into a crystalline array.

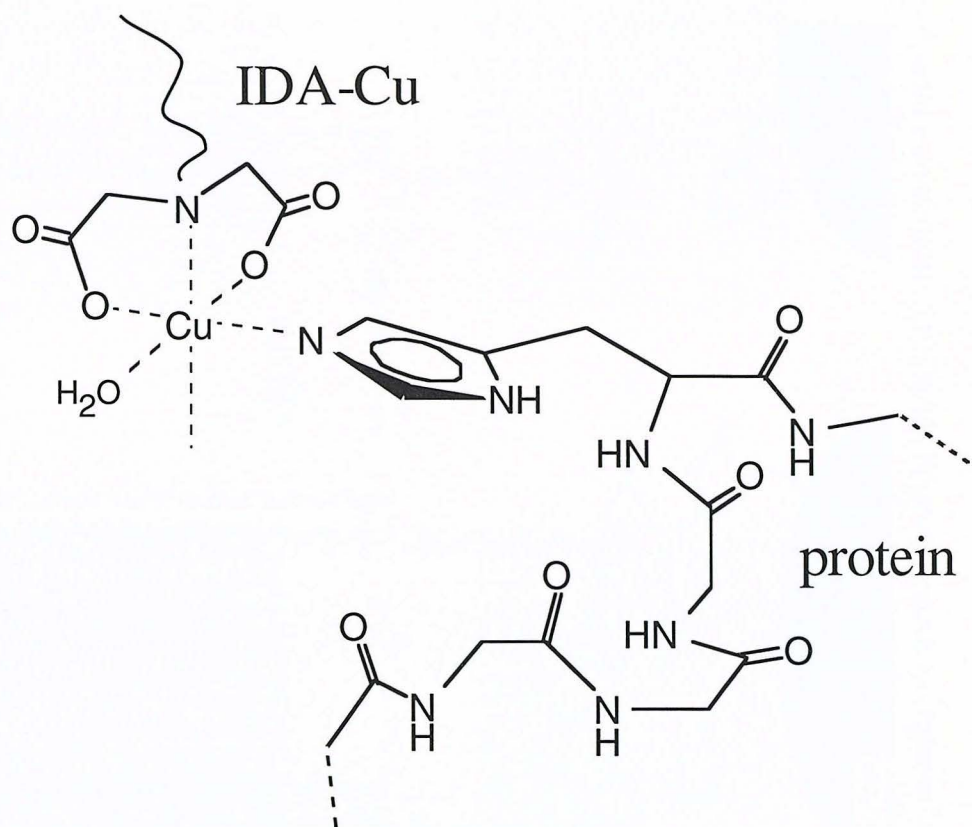


Figure 1.6. The iminodiacetate (IDA) group is a trivalent ligand, binding transition metals such as Cu^{2+} tightly yet leaving coordination sites available for binding of other ligands. The histidine side chain binds transition metal ions by coordination of the lone pair of electrons on either of the two imidazole nitrogens. The binding constant of the IDA-Cu-histidine complex is $10^{3.5} \text{ M}^{-1}$.

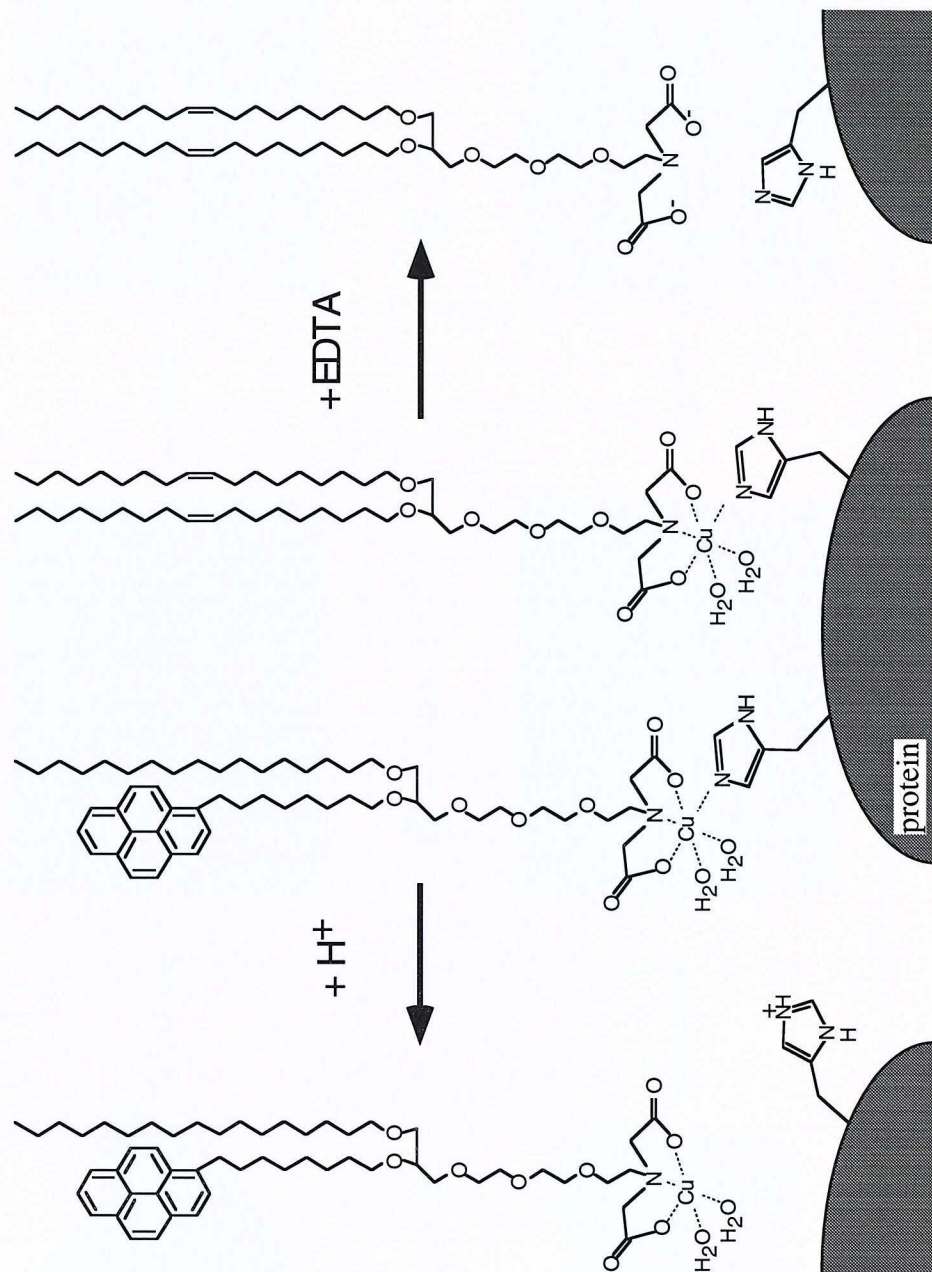


Figure 1.7. Schematic showing interaction of two representative metal-chelating lipids with histidine on a protein surface. At acidic pH, the imidazole nitrogen is protonated rendering it unable to coordinate the metal ion. The metal chelator EDTA is capable of scavenging the metal ions from the lipid headgroups, thereby disrupting the binding.

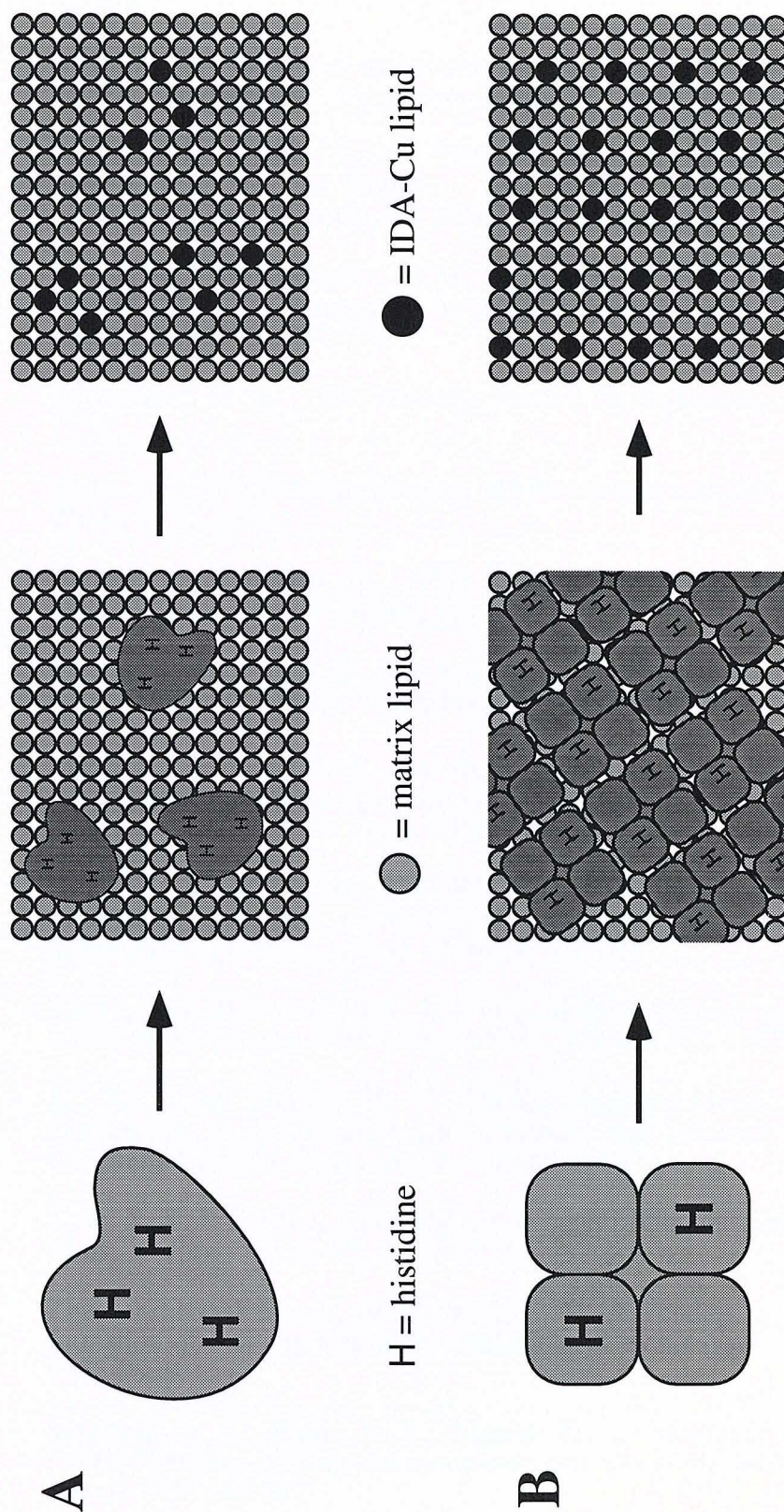


Figure 1.8. Schematic illustration of “molecular printing” of IDA-Cu lipid monolayers by histidine-containing proteins. View is looking down along the normal to the monolayer; each circle represents the cross-section of one lipid. (A) Printing of single proteins to form specific recognition sites. (B) Crystal printing results in IDA-Cu lipids patterned on the molecular scale, ordered over long distances.

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Chapter 2

Langmuir monolayer characterization of novel, metal-chelating lipids for protein targeting to membrane surfaces.

Introduction

Materials with functionalized surfaces are of importance in both materials and biological sciences for such diverse applications as construction of sensors and devices [1-3], synthesis of novel organic [4, 5] and inorganic [6-9] materials with defined, highly-ordered nano-scale architectures [10-12], catalysis [13], targeted drug delivery systems [14-16] and model studies of biological membrane recognition, organization and function [17-19]. Protein immobilization on artificial lipid membrane surfaces provides several important advantages for the synthesis of these materials. Naturally occurring proteins provide a host of functions applicable to a wide variety of problems. Nature has endowed lipids and many proteins with the ability to self-assemble into organized structures and arrays. Membranes can be composed of mixtures of lipids easily allowing the ability to tailor the density of functional sites or combine multiple functional groups on the same surface. Finally, membranes are dynamic structures capable of lateral mobility and reorganization, allowing pattern formation, enhanced binding of substrates, generation of functions and signal transduction. These various membrane properties are of major importance for a variety of applications [10, 20].

One method of targeting proteins to membrane surfaces utilizes the specific interaction between the side chain of the amino acid histidine and divalent transition metal ions [10, 21, 22] immobilized on the membrane surface. This approach has been used by our group [23-25] and others [26-28] for the attachment of various proteins and peptides to lipid monolayers. For instance, we have shown that myoglobin, containing five natural surface-accessible histidines, is targeted with moderate affinity ($K_a > 10^6 \text{ M}^{-1}$) to copper-chelating liposomes and Langmuir monolayers [23]. Cytochrome b_5 engineered to contain a hexa-histidine tag at the C-terminus was targeted to metal-chelating monolayers with somewhat higher affinity ($K_a > 2 \times 10^7 \text{ M}^{-1}$) (c.f. Chapter 3 and [24]). Poly-L-histidine was found to bind mixed IDA-Cu lipid/phosphocholine monolayers, induce aggregation of

the initially well-mixed metal-chelating lipids and, subsequently, generate an optical signal due to changes in the emission properties of the fluorescent IDA-Cu lipid [29]. Finally, the protein streptavidin was immobilized and crystallized in two-dimensions on a Langmuir monolayer *via* coordination of two of its eight naturally occurring histidines to copper-chelating lipids (c.f. Chapter 4 and [25]).

Each of these studies has relied upon synthetic lipids containing a metal-chelating moiety as the polar headgroup. The chelator immobilizes the metal ions at the aqueous surface of the membrane where it is accessible for protein binding. Several chelating lipids that can form ternary lipid-metal-protein complexes have been designed and studied in our laboratory: 1,2-distearyl-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid) (DSIDA) [23]; 1,2-dipalmityl-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid) (DPIDA); 1,2-dioleoyl-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid) (DOIDA) [25] and 1-octadecyl-2-(9-(1-pyrene)nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid) (PSIDA) [24] are shown in Figure 2.1. Variation of the ether-linked, hydrophobic tails allows desired properties to be engineered into the lipid structures (*vide infra*). Each of the lipids utilizes the tridentate metal-chelating iminodiacetate (IDA) moiety as the hydrophilic lipid headgroup. It is attached to the glycerol backbone *via* a long (ca. 11.5 Å) tri(ethylene oxide) spacer which ensures that the metal ion is accessible for protein binding. IDA forms a charge-neutral complex with divalent transition metal ions. With Cu²⁺ bound by IDA, two strong coordination sites on the metal remain available for ligand (protein) binding. The IDA-Cu complex is suitable for targeting proteins through their native surface histidines as well as through His-tags and engineered metal chelating sites. IDA can also chelate Ni²⁺, which is useful for binding His-tagged proteins.

Chelating lipids with nitrilotriacetate (NTA) headgroups have also been reported [26, 30]. This tetradentate chelating ligand is widely used with hexacoordinate Ni²⁺ for purification of His-tagged proteins [31]. The NTA-Ni complex is negatively charged, however, and high concentrations of salt (0.5 M) are usually used during protein

purification to suppress nonspecific electrostatic interactions. Because histidine forms a weaker complex with NTA-Ni than with IDA-Cu [32], NTA-Ni lipids are not suitable for targeting proteins that do not have high-affinity metal-binding sequences such as a His-tag. While this selectivity may be an advantage for chromatographic purification of His-tagged proteins, it is usually not necessary or desirable for membrane targeting of already-purified proteins.

The Langmuir monolayer properties of metal-chelating IDA lipids have been characterized at the air-water and air-buffer interfaces. Changes in surface pressure-molecular area isotherms have been used to observe chelation of Cu^{2+} from the aqueous subphase by the lipid headgroups. Epifluorescence microscopy of the Langmuir monolayers showed domain formation during compression of the DSIDA and DPIDA lipids and changes in the domain formation in the presence of copper. In addition, miscibility of the fluorescently labeled PSIDA with two common phosphocholine matrix lipids has been examined.

Results

Surface pressure-molecular area (π -A) isotherms are sensitive indicators of the molecular organization of amphiphiles at the air-water interface. The shapes of the isotherms can be affected by the presence of various molecules (e.g. metal ions, proteins) in the subphase and can provide information on ligand binding and binding-induced changes in the structure or packing of the amphiphile. We have utilized π -A isotherms to probe the ability of our IDA lipids to bind metal ions.

DSIDA.

Isotherms of monolayers of pure DSIDA with varying amounts of CuCl_2 (0-10 μM) in the buffered subphase (pH 7.5) are shown in Figure 2.2, and the characteristic

properties are summarized in Table 2.1. In the absence of metal ions, the isotherm is biphasic, exhibiting an expanded region of high compressibility with a take-off area of 140 Å²/molecule, an extended “phase transition” region (*vide infra*) between 3 and 7 mN/m, and a condensed region at high pressure with a limiting mean molecular area of 47 Å²/molecule and a collapse pressure (π_c) of 65 mN/m. With the addition of copper to the subphase, the monolayer becomes more condensed. At 0.20 μM CuCl₂, the take-off area decreases to 127 Å²/molecule, the phase transition occurs at lower pressure (2-4 mN/m), and the limiting area of the condensed phase remains unchanged. Further increasing the copper concentration to 0.35 μM leads to a complete loss of the phase transition; only the condensed phase is observed, with a limiting area of 47 Å²/molecule. Finally, at 0.50-10 μM CuCl₂, the isotherms are nearly identical to one another with limiting areas of 46 Å²/molecule and collapse pressures of 59-60 mN/m. The shapes of the isotherms at 0.50-10 μM CuCl₂ are indicative of a liquid-condensed monolayer and are similar to that observed for distearoylphosphocholine (DSPC), a natural phospholipid [33, 34]. Because the shapes of the isotherms remain unchanged, it is reasonable to assume the metal-chelating headgroups are saturated with copper at copper concentrations at or above 0.50 μM.

Fluorescence microscopy of the DSIDA monolayer (in the absence of copper) shows domain formation in the region of high compressibility at 3-7 mN/m (Figure 2.3). The fluorescent probe 1-acyl-2-(6-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)caproyl)-*sn*-glycero-3-phosphocholine (NBD-PC; 1 mol%) was included in the monolayer to allow visualization of the domains. The contrast between the dark domains and the bright background indicates a preferential partitioning of the probe lipid between the two phases [35, 36]. At high areas (100-120 Å²/molecule) small, dark domains begin to appear on the initially homogeneous bright field. Upon further compression, the domains grow in size until an area of ca. 90 Å²/molecule is reached. Beyond this point, further reduction in area

results in little growth of the domains; rather the domains tend to agglomerate at these lower areas. Ultimately, at high surface pressure (low area), the domains cover the entire surface of the monolayer (data not shown).

DPIDA.

DPIDA monolayer isotherms with varying amounts of CuCl_2 (0-10 μM) in the buffered subphase are shown in Figure 2.4 and summarized in Table 2.2. Similar to the DSIDA lipids, addition of metal ion to the subphase results in a condensation of the films. In the absence of divalent metal ions, the isotherm shows an expanded phase of high compressibility starting at an area of $133 \text{ \AA}^2/\text{molecule}$, with a phase transition at 15-19 mN/m to a more condensed phase with a limiting area of $43 \text{ \AA}^2/\text{molecule}$ and a collapse pressure of 70 mN/m. In addition, a small “kink” in the isotherm is observed at very high pressure (ca. 55 mN/m). With the addition of 0.50-10 μM CuCl_2 the isotherms become more condensed with the phase transition occurring at 6-8 mN/m, limiting areas of $41 \text{ \AA}^2/\text{molecule}$ and collapse pressures of 65 mN/m. Because the isotherms are nearly identical at these higher copper concentrations, the headgroups are again assumed to be saturated with copper ions.

Again, fluorescence microscopy allows visualization of domain formation in the “phase transition” region. Shown in Figure 2.5 are fluorescence micrographs of a DPIDA/NBD-PC (99:1) monolayer in the absence and presence of 10 μM CuCl_2 at various pressures in the phase transition region. At the onset of the phase transition in the absence of metal (Figure 2.5a), very small, dark domains appear on the bright, fluid-phase background. With further compression, the domains grow in size, but never become as large as the domains observed in the DSIDA monolayer. Again, near the end of the transition the domains tend to agglomerate. However, even as the non-compressible region of the isotherm is reached, the domains cover only a small portion of the surface area. The

same experiment was performed in the presence of 10 μM CuCl_2 (Figure 2.5b). It appears from the very low fluorescence intensities observed that the Cu^{2+} is capable of quenching the NBD-PC probe lipid, at least at high areas. Therefore, images could only be obtained near the end of the transition region. Nevertheless, we can see that the domain formation in this monolayer is different from that in the absence of metal. The dark regions are much larger and interconnect, forming a network of condensed domains even before the onset of the steep portion of the isotherm is reached (image b, Figure 2.5b). At lower molecular areas the dark regions grow to cover nearly the entire surface of the film.

DOIDA.

Isotherms of monolayers of DOIDA with varying amounts of CuCl_2 (0-10 μM) in the buffered subphase are shown in Figure 2.6 and summarized in Table 2.3. In order to check for oxidation of the unsaturated tails, π -A isotherms were measured on monolayers under both Ar and ambient atmospheres. No difference in the π -A isotherms was observed, and the experiments described herein were performed under ambient atmosphere. In contrast to the previous lipids, the DOIDA lipids remain in an expanded phase throughout the compression; no phase transition is observed at any surface pressure or any copper concentration. However, we do observe a decrease in area, at any given surface pressure, with increasing copper concentration in the subphase. Because of the high compressibility of these monolayers, the effect is much less dramatic. Adding 10 μM CuCl_2 to the subphase results in a decrease in the take-off area from 145 to 120 $\text{\AA}^2/\text{molecule}$ and a similar, yet smaller, decrease in area at all pressures (e.g., from 75 to 66 $\text{\AA}^2/\text{molecule}$ at $\pi = 20$ mN/m). Again, the isotherms at 0.50, 1.0 and 10 μM CuCl_2 are essentially identical, indicating saturation of the lipid headgroups at these copper concentrations.

PSIDA.

Isotherms of monolayers of the fluorescent PSIDA lipid are shown in Figure 2.7 and summarized in Table 2.4. In the absence of copper the monolayer exhibits a take-off area of $138 \text{ \AA}^2/\text{molecule}$ and remains relatively highly compressible at all pressures up to the collapse pressure of 39 mN/m . No phase transition is apparent, although a subtle decrease in compressibility is observed at pressures of $20\text{-}25 \text{ mN/m}$. At $0.50\text{-}10 \text{ }\mu\text{M}$ CuCl_2 , the curves are shifted to lower molecular areas with generally the same shape as in the absence of metal. The decrease in compressibility is more pronounced and occurs at lower pressure ($17\text{-}23 \text{ mN/m}$). In addition, the low compressibility region was steep enough that a limiting area of ca. $60 \text{ \AA}^2/\text{molecule}$ could be determined. Because the PSIDA lipid itself is fluorescent, the monolayer may be observed directly without the addition of an exogenous probe lipid. However, both the metalated and metal free lipids showed only a homogeneous bright field at all surface pressures when observing the pyrene monomer, excimer or total fluorescence signal (data not shown).

Changes in subphase pH also have an effect on the PSIDA isotherms, as shown in Figure 2.8. Note that these experiments were performed on a non-buffered subphase in the absence of salt and, therefore, care must be taken in comparing these isotherms to the copper binding studies above. Nonetheless, there are some interesting similarities between the pH and copper titrations of the PSIDA monolayers (cf. Tables 2.4 and 2.5). At neutral pH, the PSIDA isotherm is nearly identical to the isotherm of the fully metalated lipid on buffered subphase. Increasing to pH 10 results in an expansion of the monolayer and an isotherm nearly identical with that of the unmetalated lipid on the buffered subphase. However, decreasing the pH to 5.6 results in a slight condensation of the monolayer to areas smaller than any observed during the copper titration. Further decreasing the pH to 2.6 causes a slight expansion of the monolayer. The isotherms at pH 7.0, 5.6 and 2.6 are similar, especially at high pressures ($\pi > 20 \text{ mN/m}$).

In order to verify the role of metal chelation by the IDA headgroup in the condensation of the IDA lipid monolayers on the metal-containing subphase, two of the intermediates in the synthesis of PSIDA [24] were studied in the presence and absence of CuCl_2 . The hydroxyl terminated PS-OH (1-stearyl-2-(9-(1-pyrene)nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-ol)) and the ethyl ester analog PSIDE (1-stearyl-2-(9-(1-pyrene)nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diethyldiacetate)) are shown in Figure 2.9. These lipids are identical to PSIDA in every way except the headgroups. In contrast to the condensation of IDA lipids with the addition of CuCl_2 , the isotherms of these lipids are essentially unaffected by the presence of 10 μM CuCl_2 in the subphase (Figure 2.10 and Table 2.6).

Pre-metallated IDA lipid monolayers.

For many applications it is necessary to form a metal-containing monolayer in the absence of free Cu^{2+} in the subphase. With this in mind, we have investigated monolayers spread from a pre-metallated lipid solution; isotherms of DSIDA-Cu and DOIDA-Cu are shown as examples. The lipid headgroups were loaded with copper by adding a slight molar excess (< 10 mol%) of CuCl_2 in MeOH to the lipid solution in CHCl_3 . Isotherms of the pre-metallated lipids on subphase in the absence of metal are identical to isotherms of the same metal-free lipid spread on a subphase containing 10 μM CuCl_2 (Figure 2.11).

In addition, it is desirable to know the stability of the metal-headgroup complex; i.e. what is the lifetime of the Cu-IDA complex at the interface? Because the molecular area of the lipids at a given pressure is dependent on the amount of metal bound to the headgroup, a change in area with time can be used as a measure of the loss of metal from the lipid. The area of an un-metallated PSIDA monolayer at 15 mN/m was found to decrease by less than 5% over eight hours (Figure 2.12). This decrease in area is most likely due to evaporation of the subphase, as no attempt was made to control the humidity of the air above the trough. In contrast, pre-metallated PSIDA-Cu in the absence of free CuCl_2 in the subphase

shows an initial decrease in area, probably due to monolayer relaxation, followed by a slow increase in area. Given a difference in molecular area of $9 \text{ \AA}^2/\text{molecule}$ between the pre-metalated and metal-free lipids after eight hours, compared to a difference of $12 \text{ \AA}^2/\text{molecule}$ initially, one can roughly estimate that approximately 75% of the pre-metalated lipids contain copper after an eight hour incubation.

PSIDA/phosphocholine mixed monolayers.

Isotherms of PSIDA/DSPC mixed monolayers in the presence and absence of CuCl_2 in the subphase are shown in Figure 2.13. Pure DSPC monolayers are much more condensed than PSIDA at all pressures and show only a liquid-condensed phase with a limiting area of $47 \text{ \AA}^2/\text{molecule}$. Copper has essentially no effect on the DSPC isotherms. Both in the presence and absence of copper, the isotherms of the mixtures fall in positions intermediate between the two pure lipid monolayers, and the molecular areas increase steadily with increasing fraction of PSIDA. The one notable exception to this “ideal” behavior is the 1:1 PSIDA/DSPC mixture in which the curves (with and without copper) “cross over” at pressures higher than approximately 22 mN/m to areas lower than those of mixtures containing less PSIDA. Monolayers of DSPC withstand compression to much higher pressures than PSIDA ($\pi_c = 53 \text{ mN/m}$ for DSPC vs. 40 mN/m for PSIDA). The collapse pressures of the mixtures, with and without copper, initially increase slightly with increasing PSIDA content and, above 10 mol% PSIDA, decrease steadily toward the pure PSIDA value (Figure 2.14).

Mean molecular area plots for the mixtures are shown in Figure 2.15. The solid line drawn between the areas for the two pure components at a given pressure gives the expected area of an “ideal” mixture (or completely phase separated monolayer) with positive deviations from this line indicating repulsive interactions between the lipids and negative deviation indicating attraction. The plots show the same trends both in the presence and absence of $10 \text{ }\mu\text{M}$ CuCl_2 with only slight differences in the magnitudes of the

deviations from ideality. At low pressure, $\pi = 5$ mN/m, both plots show small positive deviations from ideality for each of the mixtures. At higher pressures, $\pi = 25$ and 35 mN/m, nearly ideal behavior is observed at low fractions of PSIDA, but negative deviations occur for mixtures containing 50 mol% or more PSIDA. A similar trend seems to appear at 15 mN/m, but the mixtures are much more near ideal at this pressure.

Mixtures of PSIDA with the fluid 1-stearoyl-2-oleoyl-phosphocholine (SOPC) were also investigated (Figure 2.16). SOPC remains in an expanded, highly compressible phase at all surface pressures. As with DSPC, the isotherms of SOPC are essentially unaffected by the presence of 10 μ M CuCl_2 . In the absence of metal, isotherms of the mixed monolayers shift away from the pure SOPC curve with increasing fraction of PSIDA. In the presence of 10 μ M CuCl_2 , however, the mixed monolayers are well-behaved at low pressures only. At pressures of 15-20 mN/m the isotherms of monolayers containing more than 30 mol% PSIDA “cross over” such that decreasing areas are observed with increasing PSIDA content at high surface pressures. The collapse pressures of the mixed monolayers, with and without copper, vary continuously with monolayer composition, showing a decreasing trend as the fraction of PSIDA is increased (Figure 2.17).

Mean molecular area plots (Figure 2.18) show nearly ideal behavior at all compositions and surface pressures, both in the presence and absence of metal ions. In the absence of copper, slight negative deviations from ideality are observed at 5 and 15 mN/m, while at 25 and 35 mN/m all data points lie on the line connecting the pure monolayer areas. With the addition of 10 μ M CuCl_2 the trends are less clear, but the mixtures appear to show small positive deviations at 15 and 25 mN/m while the areas are essentially ideal at 5 and 35 mN/m. Finally, fluorescence microscopy of the mixed monolayers (5, 10, 30 and 50 mol% PSIDA) shows a homogeneously bright field from the pyrene emission at all

surface pressures and in the presence or absence of metal, with no indication of lateral phase separation (data not shown).

Discussion

Monolayer properties of metal-chelating lipids.

Surface pressure-molecular area isotherms have been used to characterize the film-forming and metal-binding properties of four novel, iminodiacetate-containing lipids. The lipid headgroups and spacers are identical, and differences in the lipid properties must be interpreted in terms of the effect of their alkyl tails. In each case, the shapes of the isotherms are generally consistent with those of other lipids with similar tails. The long, saturated chains of DSIDA produce a condensed, solid-like film. The shorter tails of DPIDA result in a condensed state at high pressure, but a more expanded, liquid-like state at low pressures. The unsaturation of the DOIDA tails creates disorder in the lipid packing and, subsequently, an expanded phase at all pressures. In fact, it is interesting to note that the isotherms of the metalated IDA lipids are very similar in shape to isotherms of phosphocholines with the same alkyl tails [33]. This is somewhat surprising in light of the fact that (1) the IDA-Cu headgroup is somewhat larger than phosphocholine, (2) the spacer between the headgroup and glycerol backbone is longer in the IDA lipids, and (3) the tails are attached *via* ether as opposed to ester linkages.

From the shapes of the isotherms of DSIDA and DPIDA in the absence of metal and DPIDA with metal, it appears that the lipids undergo a near-constant-pressure phase transition from an expanded to a condensed state. Using fluorescence microscopy [37], we investigated the macroscopic structure of the monolayers in these regions of high compressibility by incorporating the NBD-PC lipid as a fluorescent probe. NBD-PC has been used successfully for investigating phase transitions of common phospholipids such as dipalmitoylphosphocholine (DPPC) [35, 36]. Because of the bulky fluorophore in the

lipid tail, NBD-PC preferentially partitions into the more expanded phase, preferring liquid-expanded (LE) to liquid-condensed (LC) phases [35, 36]. In each of the three systems examined here we observed the appearance of dark domains near the onset of the highly compressible region. The dark domains grew in size as the area was reduced and, for DSIDA (no metal) and DPIDA (with copper), ultimately dominated the monolayer surface. This behavior is characteristic of a phase transition from a more expanded to a more condensed phase (albeit not an ideal, first-order phase transition in which the domains grow uniformly in size throughout the transition and do not agglomerate at low areas). In addition, the non-zero slope (finite compressibility) of the phase transition regions indicates the transition is not first-order [17, 38]. The small size of the domains and their low coverage in the DPIDA monolayer, even near the onset of the LC phase, are most likely due to poor partitioning of the probe lipid between the two phases rather than incomplete transition to the condensed phase.

Copper chelation by IDA lipids.

By observing changes in the shapes of the π -A isotherms upon addition of CuCl_2 to the subphase, chelation of the metal ions by the IDA lipid headgroups could be studied. For each of the four lipids, addition of CuCl_2 induced condensation of the monolayer. Binding of metal ions to lipid interfaces has most often been shown to induce condensation [39-47] of the membranes (both monolayer and bilayer), but metal-induced expansion [44, 45, 48] has been observed as well.

The copper-induced condensation of IDA lipid monolayers saturates at CuCl_2 concentrations at and above $0.50 \mu\text{M}$; i.e. at 0.50 , 1.0 and $10 \mu\text{M}$ CuCl_2 the π -A curves are identical. Thus, it is reasonable to assume that at these concentrations the lipid headgroups are fully loaded with copper. Based on a saturation point between 0.35 and $0.50 \mu\text{M}$ CuCl_2 , one can estimate an upper limit on the apparent association constant for the lipid-copper complex of $1.3\text{-}1.8 \times 10^8 \text{ M}^{-1}$ ($\log K_{\text{Cu}} = 8.1 - 8.3$). This figure is quite low

compared to the known association constant of $10^{10.6} \text{ M}^{-1}$ for IDA and Cu^{2+} in solution [32]. One possible explanation for the large discrepancy in these binding constants could be steric hindrance caused by the large concentration of IDA at the interface; using a molecular area of $50 \text{ \AA}^2/\text{molecule}$ and a thickness of $12 \text{ \AA}$ (approximately the distance from the glycerol backbone to the chelated copper with the spacer in an extended conformation) one can estimate an IDA concentration of 2.8 M . On the other hand, the potential of the negatively charged interface (*vide infra*) should cause an enrichment of the metal ion near the monolayer and a larger apparent binding constant. This apparent contradiction will be discussed in more detail below.

The role of the IDA headgroup in the metal-induced condensation of the monolayers was confirmed by comparison of PSIDA π -A isotherms with those for the similar PS-OH and PSIDE lipids. The hydroxyl and ethyl ester headgroups of PS-OH and PSIDE, respectively, are expected to have no affinity for metal ions. Consequently the isotherms of these lipids are unaffected (within less than $1 \text{ \AA}^2/\text{molecule}$) by the addition of $10 \text{ }\mu\text{M}$ CuCl_2 (cf. Figure 2.10). This is in sharp contrast to the $10\text{-}15 \text{ \AA}^2/\text{molecule}$ reduction in area of PSIDA caused by the addition of the same amount of copper. As the chemical structures of these three lipids are identical except for the headgroups, it must be concluded that the condensation of PSIDA is induced by interaction of the Cu^{2+} with the IDA headgroup, as expected.

There are several possible explanations for the condensing effect of copper chelation by the IDA lipid headgroup. Perhaps the most obvious explanation is the neutralization of the headgroup charge upon metal chelation. In the absence of metal and on the buffered subphase the IDA headgroup is expected to carry a net charge of -1 (Figure 2.19). Chelation of the metal ion displaces a proton from the tertiary amine and results in a charge-neutral complex. Another contributing factor to the condensation could be the more compact, immobile nature of the IDA-Cu complex. Finally, there exists a possibility of

bridging of lipids by chelation of a single metal ion by two adjacent IDA lipids. However, this scenario seems unlikely as the 2:1 lipid/metal complex is highly disfavored due to the large excess of metal in the system.

The effect of subphase pH, which modulates headgroup charge, on the PSIDA isotherms appears to support the idea that monolayer condensation is due to headgroup charge neutralization. The pK_a 's of IDA in solution are well-known [32], and determination of species distributions as a function of pH is straightforward. However, as the chemical environment near charged interfaces is quite different from that in bulk solution, it is first necessary to have an understanding of the behavior of the IDA headgroups at the interface. The Gouy-Chapman-Stern theory of the diffuse double layer at charged interfaces [49, 50] provides a means of calculating the surface charge densities and species distributions of the lipid headgroups. This theory combines the Grahame equation, the Boltzmann relation and the Langmuir adsorption isotherm in order to predict the membrane surface potential and surface charge density.

The Grahame equation [50, 51] relates the surface charge density, σ , and the surface potential, Ψ_0 , according to

$$\sigma = - \left[2\epsilon_r \epsilon_0 kT \sum_i [c_i]_{\infty} \left\{ \exp \left(- \frac{z_i e \Psi_0}{kT} \right) - 1 \right\} \right]^{1/2} \quad (1)$$

where ϵ_r and ϵ_0 are the dielectric constants of water and vacuum, respectively, k is Boltzmann's constant, T is absolute temperature, e is the electronic charge and $[c_i]_{\infty}$ and z_i are the bulk concentration and valence of ionic species i , respectively. The summation is over all ionic species in solution.

The Boltzmann equation relates the concentrations of ions (protons, monovalent ions, divalent ions) at the interface, $[c_i]_0$, to the surface potential and the bulk ion concentration at infinite distance from the membrane surface

$$[c_i]_0 = [c_i]_\infty \exp\left(-\frac{z_i e \Psi_0}{kT}\right). \quad (2)$$

The Langmuir adsorption isotherms relate the surface concentrations of the various IDA lipid species and the interfacial concentrations of the cations

$$[H_3IDA^+] = K_{a3} [H_2IDA] [H^+]_0$$

$$[H_2IDA] = K_{a2} [HIDA^-] [H^+]_0 \quad (3)$$

$$[HIDA^-] = K_{a1} [IDA^{2-}] [H^+]_0$$

where $[H_3IDA^+]$, $[H_2IDA]$, $[HIDA^-]$ and $[IDA^{2-}]$ are surface concentrations of the triple-, double-, single- and non-protonated IDA species; K_{a3} , K_{a2} and K_{a1} are the interfacial protonation constants as shown in Figure 2.19; and $[H^+]_0$ is the interfacial proton concentration. The protonation constants of the IDA lipid headgroups at the interface are not known and, for the present analysis, are assumed to be equal to those for IDA in bulk solution [32]. Equations (3) can be solved for the surface charge density, σ , and related to the surface potential, Ψ_0 , by combining with equation (2) to yield

$$\sigma = \left(\frac{e}{A}\right) \frac{2 + K_{a1} [H^+]_\infty \Phi - K_{a1} K_{a2} K_{a3} [H^+]_\infty^3 \Phi^3}{1 + K_{a1} [H^+]_\infty \Phi + K_{a1} K_{a2} [H^+]_\infty^2 \Phi^2 + K_{a1} K_{a2} K_{a3} [H^+]_\infty^3 \Phi^3} \quad (4)$$

where $[H^+]_\infty$ is the known bulk proton concentration, A is the molecular area of the lipids in the monolayer and $\Phi = \exp(-e\Psi_0/kT)$. (A complete derivation of equation (4) along with a more thorough discussion of the Gouy-Chapman-Stern theory as applied to the current problem can be found in the Appendix.) Equations (1) and (4) provide two independent

equations for σ as a function of Ψ_0 and can be solved iteratively for these two parameters simultaneously.

Using this approach, the interfacial pH, PSIDA species distributions and overall surface charge have been determined for bulk subphase pH ranging from 2 to 12 (Figure 2.20). At a subphase pH of 10, where the PSIDA isotherm is relatively expanded, the theory predicts an interfacial pH of 2.3, with the negatively charged HIDA⁻ as the dominant species and an average charge per lipid of -0.40. However, at bulk pH in the range 2 to 9, where the interfacial pH is buffered at 2.0 by the IDA headgroups, the monolayer species distribution is predicted to be essentially constant. The neutral H₂IDA is the dominant species, with equal amounts of the negatively charged HIDA⁻ and positively charged H₃IDA⁺ giving a charge neutral interface. The PSIDA isotherms at pH in this range are more condensed than at pH 10 and are similar to one another in shape and area (within 2-5 Å²/molecule). This result suggests that headgroup charge neutralization may play a role in the condensation of the monolayer; negatively charged headgroups repel one another causing an expansion of the monolayer relative to neutral headgroups. Note that only a small change in interfacial pH, from pH₀ = 2.3 to 2.0, results in a large change in monolayer charge and in isotherm shape. However, given the significant variation in area between bulk pH 2.6 and 7.0, other factors such as headgroup hydration certainly contribute to the molecular areas of the lipids.

Extending the Gouy-Chapman-Stern theory to include the affinity of IDA for copper and the presence of CuCl₂ and NaCl in the subphase gives the following equation for σ (see Appendix)

$$\sigma = \frac{\left(\frac{e}{A}\right) \left(2 + (K_{a1}[H^+]_{\infty} + K_{Na}[Na^+]_{\infty})\Phi - K_{a1}K_{a2}K_{a3}[H^+]_{\infty}^3\Phi^3 \right)}{1 + (K_{a1}[H^+]_{\infty} + K_{Na}[Na^+]_{\infty})\Phi + (K_{a1}K_{a2}[H^+]_{\infty}^2 + K_{Cu}[Cu^{2+}]_{\infty})\Phi^2 + K_{a1}K_{a2}K_{a3}[H^+]_{\infty}^3\Phi^3} \quad (5)$$

where, in addition to the variable definitions given above, K_{Na} and K_{Cu} are the affinities of IDA headgroups for Na^+ and Cu^{2+} at the interface, respectively, and $[Na^+]_{\infty}$ and $[Cu^{2+}]_{\infty}$ are the ion concentrations in bulk solution. This equation allows a prediction of the amount of metalated lipid as a function of subphase copper concentration for a given value of the interfacial Cu-IDA association constant and subphase pH. In the absence of copper (on buffered subphase, pH 7.5, containing 100 mM NaCl), the theory predicts an average charge per lipid of -0.56, in comparison with -0.40 for the IDA lipid on unbuffered, no salt subphase at pH 10. This suggests the similar charges may be responsible for the similar shapes of the isotherms in these two cases. Given $\log K_{Cu} = 10.6$, the value for free IDA and Cu^{2+} in bulk solution [32], the theory (see Appendix) predicts 99% of the IDA headgroups will be metalated at copper concentrations as low as 1 nM and 100% metalated at 0.1 μM $CuCl_2$. This is inconsistent with the monolayer isotherms in which we observed saturation of the binding only at copper concentrations greater than or equal to 0.50 μM and suggests the interfacial Cu-IDA association constant is lower than that in bulk solution. Adjustment to $\log K_{Cu} = 7$ is necessary in order to predict significant amounts of unmetalated IDA at copper concentrations between 0.1 and 0.5 μM $CuCl_2$ as observed in the lipid isotherms. The $\log K_{Cu} = 6-7$ is consistent with the apparent $\log K_{Cu} = 8.1$ to 8.3 estimated above without consideration of the effect of the interfacial potential which tends to increase the concentration of Cu^{2+} near the interface. One possible explanation for the discrepancy between these interfacial binding constants and the known bulk affinity ($\log K_{Cu} = 10.6$) could be steric hindrance caused by the large concentration of IDA at the interface; using a molecular area of 50 $\text{\AA}^2/\text{molecule}$ and a thickness of 12 $\text{\AA}$ (approximately the length of the headgroup spacer) one can estimate an interfacial IDA concentration of 2.8 M.

In summary, the Gouy-Chapman-Stern theory predicts an overall charge neutral monolayer for IDA lipids on unbuffered subphase with pH_{∞} 2 to 9 and for IDA lipids fully loaded with copper on buffered subphase at pH 7.5 (with an estimated $\log K_{\text{Cu}} = 7$ and $0.50 - 10 \mu\text{M CuCl}_2$ in the subphase). Note, however, that IDA headgroups in the absence of copper are a mixture of neutral-, positive- and negative-charged groups while the copper loaded headgroups are all charge neutral. In both cases the isotherms of PSIDA are similar in shape and area. Furthermore, the theory predicts an average charge per lipid of -0.40 for IDA lipids on unbuffered subphase at pH 10 and -0.56 for IDA lipids on buffered subphase containing 100 mM NaCl at pH 7.5. Again, the isotherms in these two cases are similar in shape and area. Taken together, these results suggest the neutralization of headgroup charge is an important factor in the condensation of IDA lipid monolayers induced by copper chelation.

Mixing in PSIDA/phosphocholine lipid monolayers.

The ability to tailor the density of metal-chelating functional groups on the membrane surface could be important for a variety of applications. We have therefore studied the mixing properties of PSIDA with two neutral matrix lipids, DSPC and SOPC. These two lipids were chosen based on several criteria. First, the zwitterionic phosphocholine headgroup has only a small affinity for metal ions [52, 53] and is also inert toward binding of many proteins [17, 54, 55], which will become more important in later chapters. In addition, the stearyl and oleoyl tails, each 18 carbons in length, closely match the length of the stearyl and 9-(1-pyrene)nonyl tails of PSIDA, making miscibility of these lipids likely. Finally, the solid-condensed DSPC and highly expanded SOPC allow two very different degrees of mobility and order in the membrane.

A variety of parameters, such as molecular areas, collapse pressures, surface potential, surface viscosity [38], evaporation resistance [56] and thermodynamic properties

[57-59], can be used to characterize the degree of mixing in two-component monolayers. In the present work we have utilized the molecular areas, collapse pressures and calculated thermodynamic properties to investigate the mixing of the PSIDA/DSPC and PSIDA/SOPC systems, in the absence and presence of copper. For instance, collapse pressures of miscible monolayers are expected to vary continuously with monolayer composition, while for a phase-separated system the entire mixed monolayer will show a collapse pressure characteristic of the less stable (lower collapse pressure) component [38]. The continuous variation of collapse pressures in both PSIDA/DSPC and PSIDA/SOPC mixtures, in the absence and presence of metal ions, suggests that PSIDA is miscible with the phosphocholine lipids.

It is common to analyze mixed monolayer isotherms in terms of an excess area of mixing, which represents the difference in packing efficiency of the mixture components over the additive (“ideal”) mixing values in which no interactions are present between the two components. The excess area, $\Delta A_{\text{mix}}^E(\pi)$, is given by

$$\Delta A_{\text{mix}}^E(\pi) = A_{12}(\pi) - x_1 A_1(\pi) - x_2 A_2(\pi) \quad (6)$$

where $A_{12}(\pi)$ is the mean molecular area of the mixture at a given surface pressure, $A_1(\pi)$ and $A_2(\pi)$ are the molecular areas of the pure components at the same pressure, and x_1 and x_2 are the mole fractions of components 1 and 2, respectively. When $\Delta A_{\text{mix}}^E = 0$ either ideal mixing or complete phase separation is indicated; i.e. there is no interaction between the lipid components. $\Delta A_{\text{mix}}^E < 0$ indicates a more efficient packing of, or attraction between, the lipids, while $\Delta A_{\text{mix}}^E > 0$ indicates packing constraints or less efficient packing in the mixture. It is important to note, however, that $\Delta A_{\text{mix}}^E \neq 0$ indicates an interaction between the lipid components and, therefore, some degree of miscibility.

The excess area is merely a replotting of the mean molecular area diagrams (cf. Figures 2.15 and 2.18), but in this instance allows an easy comparison of the mixed

monolayers in the presence and absence of copper. The PSIDA/DSPC monolayers show large positive values of ΔA_{mix}^E at low surface pressure (Figure 2.21). As the pressure is increased, ΔA_{mix}^E moves toward zero at less than 35 mol% PSIDA, while negative values of ΔA_{mix}^E are observed at larger PSIDA fractions. Within the experimental error, essentially no difference was observed upon addition of 10 μM CuCl_2 to the subphase.

The excess areas are smaller overall for the PSIDA/SOPC mixtures (Figure 2.22). In the absence of metal, ΔA_{mix}^E values are less than zero at low surface pressure and move toward zero with increasing pressure. Different behavior is observed in the presence of 10 μM CuCl_2 , however. Again, values of ΔA_{mix}^E are negative at low surface pressures and low PSIDA content. Increasing surface pressure to 15 and 25 mN/m results in ΔA_{mix}^E greater than zero, and, at 35 mN/m, the excess areas return to zero. In contrast to the PSIDA/DSPC system, the PSIDA/SOPC ΔA_{mix}^E are very different in the absence and presence of copper, except that $\Delta A_{\text{mix}}^E = 0$ at very high surface pressure, irrespective of metal content.

It is common practice in the study of bulk solutions to characterize mixtures in terms of excess thermodynamic properties. In two-dimensional solutions, we can apply the same definitions and derivations to the problem of mixing at interfaces. In fact, Goodrich has developed a method for calculating excess free energies of mixing from the monolayer surface pressure-molecular area isotherms [57]. The free energy is defined as

$$G = U - TS + PV - \gamma A \quad (7)$$

and the total differential is then

$$dG = -SdT + VdP - Ad\gamma + \sum \mu_i dn_i \quad (8)$$

where S is entropy; T , temperature; V , volume; P , pressure; A is the area of the interface; γ is surface tension; μ , chemical potential; n , moles and the summation extends over all chemical species, i , in the system. In a typical Langmuir monolayer experiment, all variables are constant except for the surface tension, and equation (8) reduces to

$$dG = -Ad\gamma. \quad (9)$$

Integration of equation (9) leads to

$$\Delta G_{\text{mix}}^{\text{tot}} = \Delta G_{\text{mix}}^{\text{id}} + \Delta G_{\text{mix}}^{\text{E}} \quad (10)$$

where $\Delta G_{\text{mix}}^{\text{id}}$ is the normal statistical energy of ideal mixing, $RT(x_1 \ln x_1 + x_2 \ln x_2)$, and $\Delta G_{\text{mix}}^{\text{E}}$ is the excess free energy of mixing calculated from experimentally accessible parameters according to

$$\Delta G_{\text{mix}}^{\text{E}} = \int_{\pi^*}^{\pi} A_{12} d\pi - x_1 \int_{\pi^*}^{\pi} A_1 d\pi - x_2 \int_{\pi^*}^{\pi} A_2 d\pi. \quad (11)$$

A_{12} is the molecular area of the mixed monolayer, A_1 and A_2 are the molecular areas of the pure monolayers, and x_1 and x_2 are the mole fractions of each of the two components in the mixture. The integrals equate to the areas under the π - A isotherms, and π^* is defined as the pressure where the monolayer components are ideally miscible. It is generally assumed that $\pi^* \approx 0$. In practice, π^* is commonly set to the lowest measurable surface pressure [58, 59], and in this work π^* was set to 0.05 mN/m. However, Pagano and Gershfeld [60] have shown that the very-low-pressure, high-molecular-area portion of the isotherm can contribute significantly to the integrals in equation (11), and underestimation of the free energy of mixing may result if data are not obtained to tens of thousands of $\text{\AA}^2/\text{molecule}$. Since obtaining data at these large areas is impractical with most experimental set-ups (including ours), the possible errors are necessarily ignored.

As with ΔA_{mix}^E , $\Delta G_{\text{mix}}^E = 0$ is defined as “ideal” mixing. Negative deviations indicate the solution is thermodynamically more stable than the unmixed state and are generally an indication of miscibility. Positive deviations indicate some packing constraints in the mixed system and suggest the possibility of phase separation. However, one must determine that the total free energy of mixing, $\Delta G_{\text{mix}}^{\text{tot}}$, is greater than zero before phase separation can be concluded.

Figure 2.23 and Tables 2.7 and 2.8 compare the calculated ΔG_{mix}^E in the absence and presence of 10 μM CuCl_2 for the PSIDA/DSPC mixed monolayers at various pressures. At low pressures, positive values of ΔG_{mix}^E are observed in the absence of copper which increase in magnitude as the pressure is increased to 15 mN/m and then decrease as the pressure is further increased to 25 and 35 mN/m. In the presence of copper, nearly the same trend is observed with slightly larger values of ΔG_{mix}^E at high pressures. These positive values of ΔG_{mix}^E could indicate partial phase separation. However, the negative values of $\Delta G_{\text{mix}}^{\text{tot}}$ for all mixtures and all compositions, with and without copper, (Tables 2.7 and 2.8) make phase separation unlikely. Similar data for the PSIDA/SOPC system are shown in Figure 2.24 and Tables 2.9 and 2.10. In the absence of metal, the mixtures are ideal at low pressure with negative values of ΔG_{mix}^E at 15 and 25 mN/m and large positive ΔG_{mix}^E at 35 mN/m. In the presence of copper, the mixtures are essentially ideal at 5, 15 and 25 mN/m with small, positive values of ΔG_{mix}^E at 35 mN/m. Again, $\Delta G_{\text{mix}}^{\text{tot}}$ is less than zero for most mixtures and pressures with the exception of low mole fraction PSIDA mixtures with no metal at 35 mN/m. Phase separation of PSIDA/SOPC mixtures at 35 mN/m is likely.

The excess areas and free energies of mixing provide complementary views of mixing in the lipid monolayers. The ΔA_{mix}^E values depend only on the final molecular areas and represent the final efficiency in packing of the two lipids at a fixed pressure. In contrast, ΔG_{mix}^E values are calculated as the area under the isotherm and, as such, are

highly dependent on the shape of the π -A curves. These values represent the spontaneity of mixing of the two lipids up to a given pressure. Combining these data, a physical picture of the interactions between the lipid components in the monolayer mixtures can be developed.

At low pressure, the positive values of ΔA_{mix}^E for the PSIDA/DSPC system show the difficulty of packing the bulky PSIDA in the liquid-condensed phase of the DSPC matrix. The packing constraints are alleviated somewhat at increased pressures as the PSIDA itself becomes more condensed. In fact, at high pressures and high PSIDA mole fraction, the negative deviations in ΔA_{mix}^E demonstrate the efficient packing of the highly condensed DSPC lipids into the PSIDA matrix. The difficulty of squeezing PSIDA into the well-packed, solid-like DSPC is reflected in the positive values of ΔG_{mix}^E . In comparison, the more expanded, disordered SOPC lipid allows efficient packing with the PSIDA lipids as evidenced by the negative values of ΔA_{mix}^E . However, chelation of copper by the IDA headgroup disrupts the packing in these monolayers as evidenced by the positive ΔA_{mix}^E . The zero and slightly negative values of ΔG_{mix}^E at 5, 15 and 25 mN/m indicate these mixtures are stable. In contrast, at $\pi = 35$ mN/m, positive values of ΔG_{mix}^E were calculated with very large values in the absence of metal. In fact, $\Delta G_{\text{mix}}^{\text{tot}}$ in the absence of metal at 35 mN/m is positive for 5 and 10 mol% PSIDA and zero, within error, at compositions containing more PSIDA suggesting phase separation at this high pressure. In summary we can conclude, based on the calculated ΔA_{mix}^E , ΔG_{mix}^E , and $\Delta G_{\text{mix}}^{\text{tot}}$, that the PSIDA is generally miscible with both DSPC and SOPC (with the limited exceptions noted above), both in the absence of metal and when loaded with copper.

The miscibility of PSIDA with phosphocholines is important in many applications. One of the most notable is the fluorescent metal ion sensor based on PSIDA/DSPC (1:19) mixed liposomes [3]. This sensor relies on a dramatic decrease in the ratio of excimer to monomer emission intensities (E/M) upon binding of metal ions to the PSIDA lipid headgroups. Pyrene E/M is directly proportional to the collision frequency of pyrene lipids

and as such depends on the local concentration of PSIDA as well as its rate of lateral diffusion in the membrane [61]. In the metal sensor, the large effect of metal binding on E/M is believed to be due to a change in local PSIDA concentration; i.e. metal binding induces a dispersion of partially phase-separated PSIDA aggregates [3]. This supposition is supported by studies of other PSIDA/phosphocholine mixed liposomes which showed that the decrease in E/M upon metal binding correlated with the difference in melting transition temperature of PSIDA and the matrix lipid [62]. Another possible mechanism for the E/M decrease which must be considered is quenching of the pyrene fluorescence by the copper ions. However, quenching seems unlikely as the E/M decrease could be effected by the addition of metal ions such as Ca^{2+} and Zn^{2+} which are not expected to show quenching of pyrene.

In monolayers, copper does not affect mixing in the PSIDA/DSPC system; metalated and unmetalated PSIDA interact equally with DSPC as measured by collapse pressures, excess areas and free energies of mixing. The PSIDA/SOPC system, which showed little effect of copper in the vesicles, was also found to be miscible at most pressures and PSIDA mole fractions. However, the interactions between PSIDA and SOPC are significantly different in the absence and presence of copper, as described above. Care must be taken when extrapolating results obtained from monolayer systems to bilayer systems such as vesicles [17]. Membrane curvature is known to have significant effects on headgroup interactions, packing of the hydrophobic tails and, subsequently, membrane mixing [63]. Nonetheless, the present results appear to contradict the supposition of metal-induced aggregate dispersion in the vesicles. This discrepancy is not clearly understood.

Conclusions

The metal-chelating IDA lipids form stable Langmuir monolayers at the air-water and air-buffer interfaces. Each of the lipids binds copper in the subphase, becoming

saturated with metal at 0.35 to 0.50 μM CuCl_2 (apparent $\log K_a = 8.1$ to 8.3). This much weaker binding than for free IDA and Cu^{2+} in solution ($\log K_a = 10.6$) is most likely due to steric hindrance in the large local concentration of IDA at the monolayer surface. The variation of the alkyl tails allows for a wide variety of monolayer properties. DSIDA provides a condensed, rigid monolayer while DOIDA allows for more fluid films. The fluorescently labeled PSIDA provides a probe for investigating the environment of the IDA lipid in the membrane.

The fluorescence properties of PSIDA make this lipid a powerful tool for basic studies of ligand binding to metal-chelating membrane surfaces as well as a wide variety of applications [20, 29]. This lipid and its mixtures with phosphocholine matrix lipids have therefore been studied in greater detail. PSIDA was found to be generally miscible with both DSPC and SOPC, both in the absence and presence of metal, allowing the possibility to easily tailor the density of functional lipids on the membrane surface while controlling the fluidity and organization of the membrane as a whole. PSIDA and PSIDA-Cu show essentially no differences in their interactions with DSPC. However, the unmetalated and metalated lipids do show differences in their interactions with SOPC. The unmetalated lipid packs efficiently with SOPC at low pressures but appears to phase separate at high pressures, while PSIDA-Cu shows some packing constraints with SOPC at low pressure but appears well-mixed at all pressures.

The various IDA lipids provide us with a simple, robust material for the general and convenient production of new functionalized surfaces. Self-assembled lipid membranes have several advantages for the synthesis of such materials including their inherent organization and dynamic nature. The metal-chelating headgroups allow decoration of the membrane surface with a wide variety of proteins based on their histidine content. Both naturally occurring and engineered histidine residues should extend this approach to almost any protein of interest.

Experimental

Materials.

The metal chelating lipids DSIDA [23], PSIDA [24] and DOIDA [25, 64] were synthesized as described. The synthesis of DPIDA followed the same scheme and will be described elsewhere. All lipid syntheses were performed within our group by Dr. Darryl Y. Sasaki, Dr. Chao-Tsen Chen and Dr. Guohua Chen. The phosphocholines 1,2-distearoyl-*sn*-3-glycero-phosphocholine (DSPC), 1-stearoyl-2-oleoyl-*sn*-3-glycero-phosphocholine (SOPC) and 1-acyl-2-(6-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)caproyl)-*sn*-glycero-3-phosphocholine (NBD-PC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and were used as received without further purification. Chloroform, methanol, ethanol (all A.C.S. HPLC grade) and CuCl₂ (99.999%) were obtained from Aldrich (Milwaukee, WI). 3-(N-morpholino)propanesulfonic acid (MOPS) was obtained from Sigma (St. Louis, MO).

Langmuir monolayer measurements.

Monolayer studies were carried out on a computer-controlled Langmuir film balance (M1200, KSV Instruments, Helsinki) with a surface area of 250 x 75 mm² and equipped with two movable barriers. The entire film balance was placed inside a Plexiglas box and was thermostatted by circulating water through the metal base of the trough. The surface pressure was measured by the Wilhelmy plate method using 1 x 2 cm² plates cut from filter paper (Whatman, No. 1). The plate was rinsed thoroughly with chloroform, methanol and water, and was periodically calibrated with stearic acid using the transition at 25 mN/m and 25 °C.

Lipid solutions were prepared in chloroform. Mixed monolayers were prepared by mixing appropriate volumes of stock solutions prior to spreading. Pre-metalated IDA lipids were prepared by adding an aliquot of concentrated CuCl₂ (ca. 10% molar excess Cu²⁺) in methanol to the lipid solutions. Purity of the lipids was confirmed by thin layer

chromatography using silica gel glass plates (E. Merck, Darmstadt, Germany). The plates were developed using chloroform/methanol/water (65:25:4) and all lipids migrated as a single spot.

The subphase was buffered to slightly alkaline pH (20 mM MOPS, 100 mM NaCl, pH 7.5), except where noted, to facilitate deprotonation of the IDA headgroups and subsequent binding of metal ions. All subphase solutions were prepared with Milli-Q purified water (18.2 M Ω ·cm; Milli-Q UV Plus, Millipore Corp., Bedford, MA). In order to remove contaminating metals such as Ca²⁺, the buffer was stirred for ~30 min. with ~1 g of Chelex 100 (Sigma), an IDA-derivatized chromatography resin, and then filtered to remove the Chelex beads. Monolayers were spread dropwise using a Hamilton syringe and, after allowing 15 min. for solvent evaporation, were compressed at a constant rate of 3 Å²/molecule·min until film collapse was observed. All isotherms were reproducible to < 1 Å²/molecule. Between experiments the trough was cleaned with ethanol and chloroform followed by copious rinsing with water. Take-off area is defined as the molecular area at which the surface pressure increases from its constant, small value observed at high areas. Limiting areas were determined as the point of intersection of the abscissa and the tangent drawn to the steepest portion of the isotherm.

Fluorescence microscopy of Langmuir monolayers.

A circular quartz window (~ 5 cm in diameter) was incorporated into the bottom of the Langmuir trough, and the entire Langmuir instrument was placed above the stage of an inverted epifluorescence microscope (Zeiss Axiovert 35). The experiment was performed as described above except the compression speed was typically much slower: 0.5-1 Å²/molecule·min. An HBO 100 W/2 Hg arc lamp was used as the excitation source. A long-working-distance reflecting objective (36x, NA 0.5, 8 mm working distance; Ealing Electro-Optics, Holliston, MA), transmitting light from the far UV to near IR, allows UV

excitation of the pyrene fluorophore. Excitation and emission wavelengths were selected using appropriate narrow-band interference filters. The NBD-PC lipid was imaged using excitation in the range 450-490 nm and a 515-565 nm band pass emission filter (Zeiss set 487910). Pyrene fluorescence was excited by selection of the 334 nm line of the Hg lamp *via* a narrow bandpass interference filter (10 nm FWHM; Melles Griot, Irvine, CA). Using appropriate filters, it is possible to selectively observe pyrene monomer and excimer fluorescence (405DF35 and 470DF40, respectively; Omega Optical, Inc., Brattleboro, VT). The images were obtained *via* a variable-gain image intensifier (Hamamatsu C2400-68) with a solid-state CCD camera (Hamamatsu C2400-60) and were recorded on a video cassette recorder or digitized directly using a frame grabber (AG-5, Scion Corp., Frederick, MD) on a Macintosh compatible computer (Power 100, Power Computing, Austin, TX). (The AG-5 frame grabber allows multiple frames to be averaged improving image quality of low contrast, low light samples. The images presented are most commonly averages of five consecutive frames; however, some images are averages of up to 50 frames.) Images were manipulated using the program NIH Image (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

CuCl ₂ (μ M)	take-off area ($\text{\AA}^2/\text{molecule}$)	limiting area ($\text{\AA}^2/\text{molecule}$)	collapse pressure (mN/m)	phase transition region (mN/m)
0	138	47	65	3-7
0.20	127	47	65	2-4
0.35	72	47	60	-
0.50	58	46	59	-
1.0	56	46	59	-
10	54	46	60	-

Table 2.1. Characteristic properties of pure DSIDA monolayer π -A isotherms on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5) at 25 °C in the presence of varying concentrations of CuCl₂.

CuCl ₂ (μ M)	take-off area ($\text{\AA}^2/\text{molecule}$)	limiting area ($\text{\AA}^2/\text{molecule}$)	collapse pressure (mN/m)	phase transition region (mN/m)
0	133	43	70	15-19
0.50	112	42	65	6-8
1.0	113	42	65	6-8
10	111	41	65	6-7

Table 2.2. Characteristic properties of pure DPIDA monolayer π -A isotherms on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5) at 25 °C in the presence of varying concentrations of CuCl₂.

CuCl ₂ (μ M)	take-off area ($\text{\AA}^2/\text{molecule}$)	area at 20 mN/m ($\text{\AA}^2/\text{molecule}$)	collapse pressure (mN/m)
0	145	75	41
0.20	132	70	43
0.35	127	69	43
0.50	122	67	43
1.0	120	66	42
10	124	68	42

Table 2.3. Characteristic properties of pure DOIDA monolayer π -A isotherms on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5) at 25 °C in the presence of varying concentrations of CuCl₂.

CuCl_2 (μM)	take-off area ($\text{\AA}^2/\text{molecule}$)	limiting area ($\text{\AA}^2/\text{molecule}$)	collapse pressure (mN/m)
0	138	ND	39
0.50	122	61	40
1.0	124	60	40
10	124	59	41

Table 2.4. Characteristic properties of pure PSIDA monolayer π -A isotherms on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5) at 25 °C in the presence of varying concentrations of CuCl_2 .

pH	take-off area ($\text{\AA}^2/\text{molecule}$)	limiting area ($\text{\AA}^2/\text{molecule}$)	collapse pressure (mN/m)
10	138	ND	39
7.0	126	58	41
5.6	118	56	40
2.6	126	59	40

Table 2.5. Characteristic properties of pure PSIDA monolayer π -A isotherms as a function of pH at 25 °C. Subphase is pure water adjusted to appropriate pH with either HCl or NaOH.

	CuCl ₂ (μ M)	take-off area ($\text{\AA}^2/\text{molecule}$)	limiting area ($\text{\AA}^2/\text{molecule}$)	collapse pressure (mN/m)	phase transition (mN/m)
PSIDA	0	138	ND	39	60
	10	124	59	41	74
PSIDE	0	196	ND	23	67
	10	196	ND	23	67
PS-OH	0	123	59	35	52
	10	118	58	35	50

Table 2.6. Characteristic properties of PSIDA monolayer π -A isotherms and the synthetic intermediates PS-OH and PSIDE in the absence of metal ion and presence of 10 μ M CuCl₂ on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5) at 25 °C.

		surface pressure (mN/m)						
x_{PSIDA}		5	10	15	20	25	30	35
$\Delta G^{\text{E}}_{\text{mix}}$ (J/mol)	0.05	47.6	-94.8	-50	-278	-202	-108	-301
	0.10	282	244	411	257	560	506	181
	0.25	473	477	728	646	677	843	542
	0.50	379	545	626	430	391	181	-60.2
	0.75	250	414	403	215	105	-60.2	-60.2
$\Delta G^{\text{tot}}_{\text{mix}}$ (J/mol)	0.05	-445	-587	-543	-770	-694	-601	-793
	0.10	-524	-562	-396	-549	-246	-300	-626
	0.25	-922	-918	-666	-749	-717	-552	-852
	0.50	-1340	-1170	-1090	-1290	-1330	-1540	-1780
	0.75	-1140	-981	-991	-1180	-1290	-1460	-1460

Table 2.7. Excess and total free energies of mixing for the PSIDA/DSPC monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C) in the absence of metal ion at various surface pressures. Excess free energies were calculated according to equation (11). Total free energies are the sum of the excess free energies and the ideal free energy of mixing, $RT(x_1 \ln x_1 + x_2 \ln x_2)$. For the reader's convenience, positive energies are shaded.

		surface pressure (mN/m)						
	x_{PSIDA}	5	10	15	20	25	30	35
$\Delta G_{\text{mix}}^{\text{E}}$ (J/mol)	0.05	86.1	31.3	137	51.2	-36.1	21.1	54.2
	0.10	312	256	423	257	259	445	120
	0.25	334	608	638	527	783	527	632
	0.50	494	674	734	632	722	452	421
	0.75	310	512	548	497	421	436	271
$\Delta G_{\text{mix}}^{\text{tot}}$ (J/mol)	0.05	-406	-461	-355	-441	-528	-471	-438
	0.10	-494	-550	-384	-549	-547	-361	-686
	0.25	-1060	-787	-756	-868	-612	-868	-762
	0.50	-1220	-1040	-985	-1090	-997	-1270	-1300
	0.75	-1080	-883	-847	-898	-973	-958	-1120

Table 2.8. Excess and total free energies of mixing for the PSIDA/DSPC monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C) in the presence of 10 μM CuCl_2 at various surface pressures. Excess free energies were calculated according to equation (11). Total free energies are the sum of the excess free energies and the ideal free energy of mixing, $RT(x_1 \ln x_1 + x_2 \ln x_2)$. For the reader's convenience, positive energies are shaded.

		surface pressure (mN/m)							
		x _{PSIDA}	5	10	15	20	25	30	35
$\Delta G^{\text{E}}_{\text{mix}}$ (J/mol)	0.05	-32.2	-61.7	-42.1	-256	-93.3	-409	1690	
	0.10	-58.4	-160	-205	-331	-427	626	1700	
	0.30	-72.8	-160	-253	-331	-440	373	1440	
	0.50	-21.1	-208	-181	-211	-271	-361	1720	
	0.70	-23.5	-172	-169	-90.3	18.1	530	1390	
	0.90	-13.8	-178	-36.1	-150	-114	217	945	
	0.95	-52.1	-179	-199	-226	-328	-494	-69.2	
$\Delta G^{\text{tot}}_{\text{mix}}$ (J/mol)	0.05	-524	-554	-534	-748	-586	-902	1200	
	0.10	-865	-966	-1010	-1140	-1230	-108	897	
	0.30	-1590	-1670	-1770	-1850	-1950	-1140	-76.2	
	0.50	-1740	-1930	-1900	-1930	-1990	-2080	-3.3	
	0.70	-1540	-1690	-1680	-1600	-1500	-985	-124	
	0.90	-820	-984	-842	-957	-921	-590	139	
	0.95	-544	-671	-691	-718	-820	-986	-562	

Table 2.9. Excess and total free energies of mixing for the PSIDA/SOPC monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C) in the absence of metal ion at various surface pressures. Excess free energies were calculated according to equation (11). Total free energies are the sum of the excess free energies and the ideal free energy of mixing, $RT(x_1 \ln x_1 + x_2 \ln x_2)$. For the reader's convenience, positive energies are shaded.

		surface pressure (mN/m)						
x_{PSIDA}		5	10	15	20	25	30	35
$\Delta G_{\text{mix}}^{\text{E}}$ (J/mol)	0.05	-78.3	-15.6	21.1	-162	-166	-211	90.3
	0.10	134	292	397	391	415	385	680
	0.30	-96.3	2.41	-54.2	48.2	30.1	181	241
	0.50	-120	-138	-30.1	0	90.3	241	181
	0.70	-36.1	-56.6	-6.02	193	211	301	301
	0.90	18.1	152	138	265	271	482	421
	0.95	-18.1	15.7	99.3	163	-15.0	391	30.1
$\Delta G_{\text{mix}}^{\text{tot}}$ (J/mol)	0.05	-571	-508	-471	-655	-658	-703	-402
	0.10	-672	-514	-409	-415	-391	-421	-126
	0.30	-1610	-1510	-1570	-1470	-1480	-1330	-1270
	0.50	-1840	-1860	-1750	-17200	-1630	-1480	-1540
	0.70	-1551	-1572	-1521	-1322	-1304	-1214	-1214
	0.90	-788	-654	-668	-541	-535	-325	-385
	0.95	-510	-477	-393	-330	-507	-101	-462

Table 2.10. Excess and total free energies of mixing for the PSIDA/SOPC monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C) in the presence of 10 μM CuCl_2 at various surface pressures. Excess free energies were calculated according to equation (11). Total free energies are the sum of the excess free energies and the ideal free energy of mixing, $RT(x_1 \ln x_1 + x_2 \ln x_2)$. For the reader's convenience, positive energies are shaded.

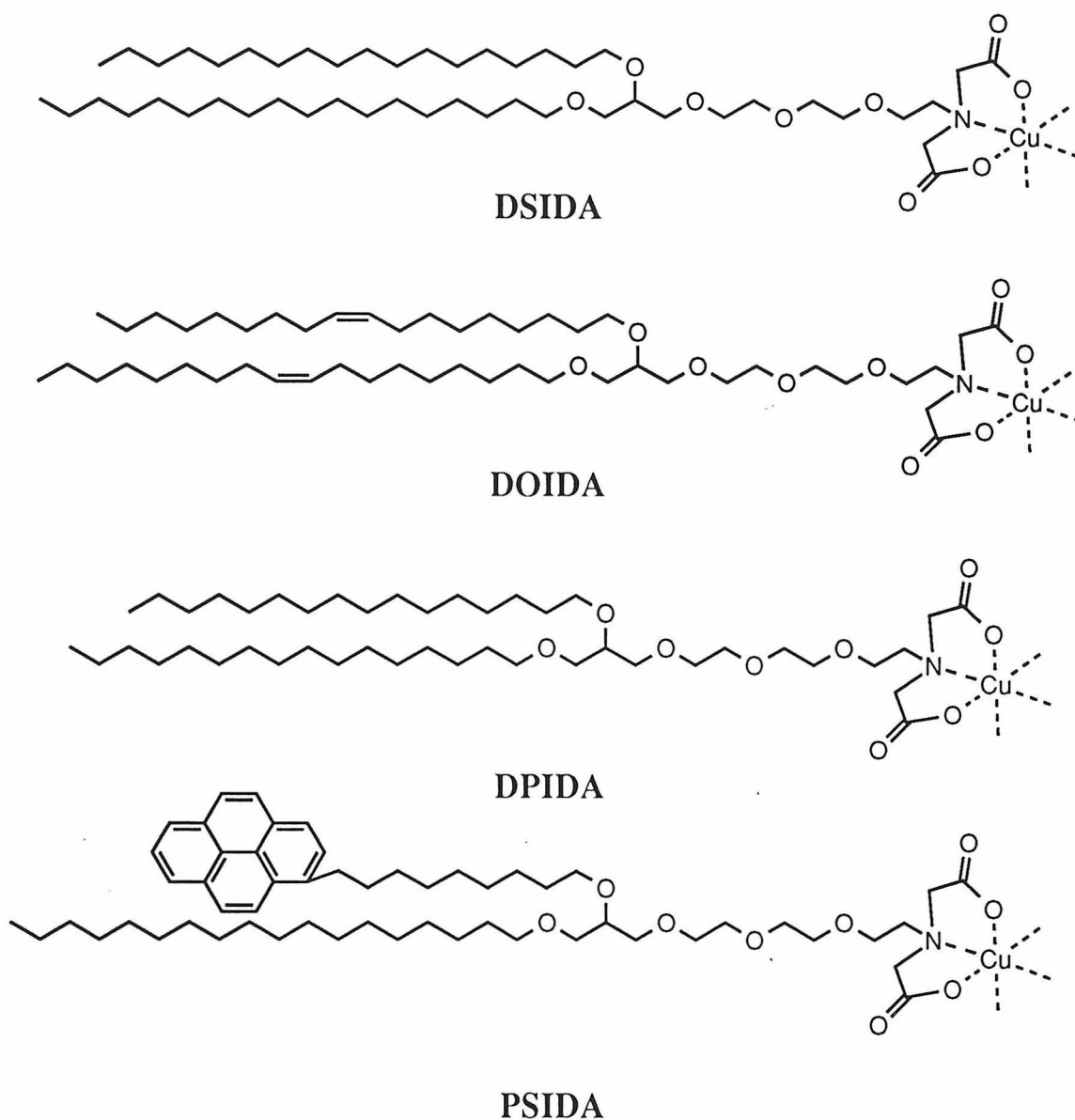


Figure 2.1. Structures of synthetic metal chelating lipids 1,2-distearyl-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-*N,N*-diacetic acid) (DSIDA); 1,2-dipalmityl-*rac*-glycero-3-(8-(3,6-dioxy)-octyl-1-amino-*N,N*-diacetic acid) (DPIDA); 1,2-dioleyl-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-*N,N*-diacetic acid) (DOIDA); and 1-stearyl-2-(9-(1-pyrene)-nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-*N,N*-diacetic acid) (PSIDA) shown as the copper complex.

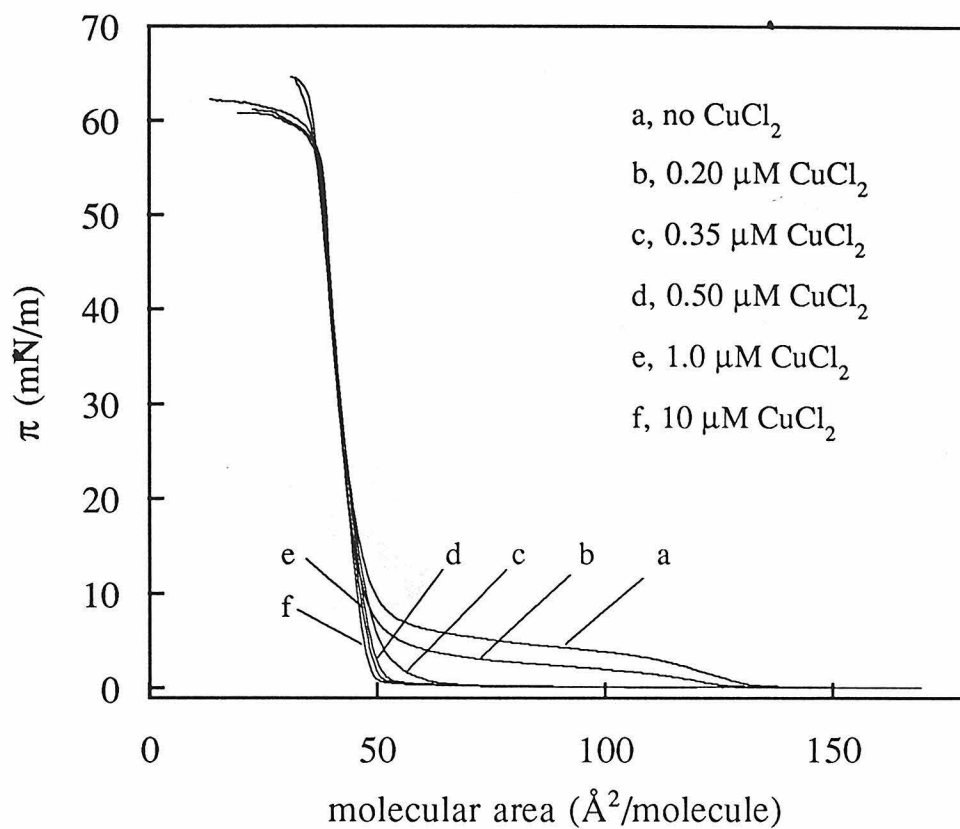


Figure 2.2. Surface pressure-molecular area isotherms of pure DSIDA lipid in the presence of varying amounts of CuCl_2 ; curve a, no divalent metal; b, $0.20 \mu\text{M}$ CuCl_2 ; c, $0.35 \mu\text{M}$ CuCl_2 ; d, $0.50 \mu\text{M}$ CuCl_2 ; e, $1.0 \mu\text{M}$ CuCl_2 ; and f, $10 \mu\text{M}$ CuCl_2 on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25°C).

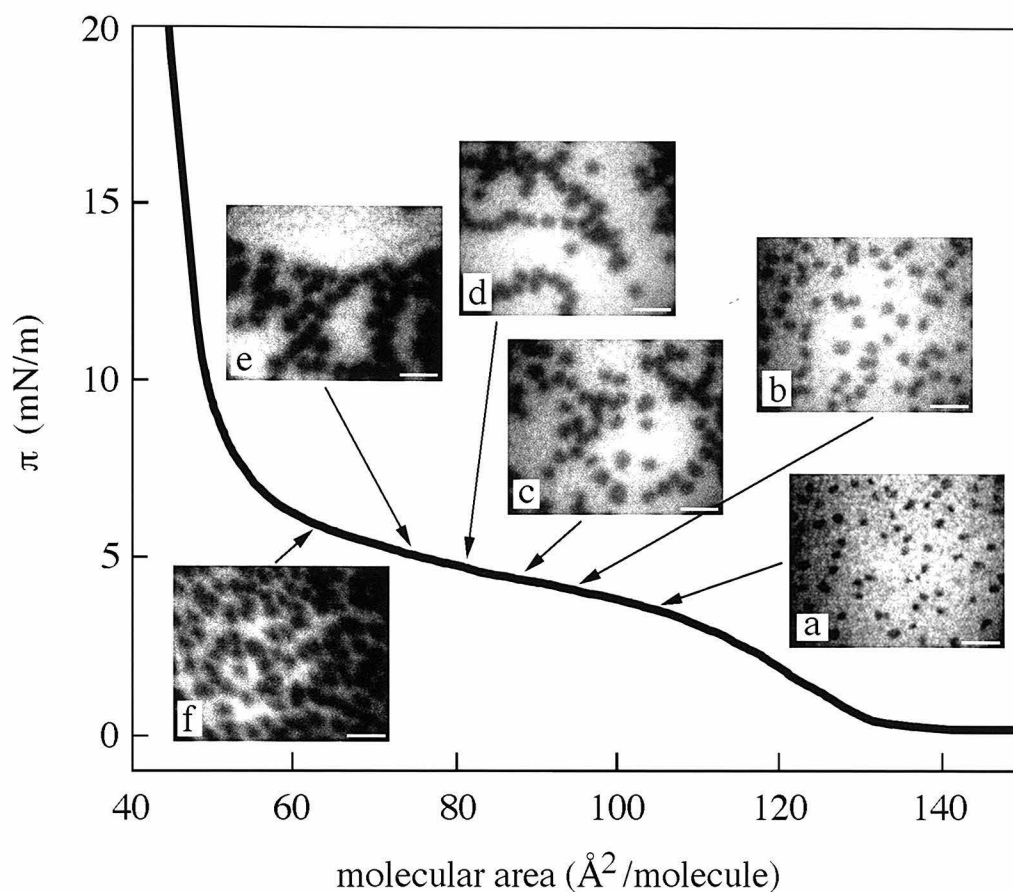


Figure 2.3. Fluorescence micrographs of DSIDA/NBD-PC (99:1) monolayer at various points throughout the phase transition (a, $\pi = 3.6$ mN/m; b, 4.1 mN/m; c, 4.4 mN/m; d, 4.7 mN/m; e, 5.0 mN/m; f, 6.0 mN/m). Subphase is 20 mM MOPS, 100 mM NaCl, pH 7.5 at room temperature. Scale bar in each image is 50 μm .

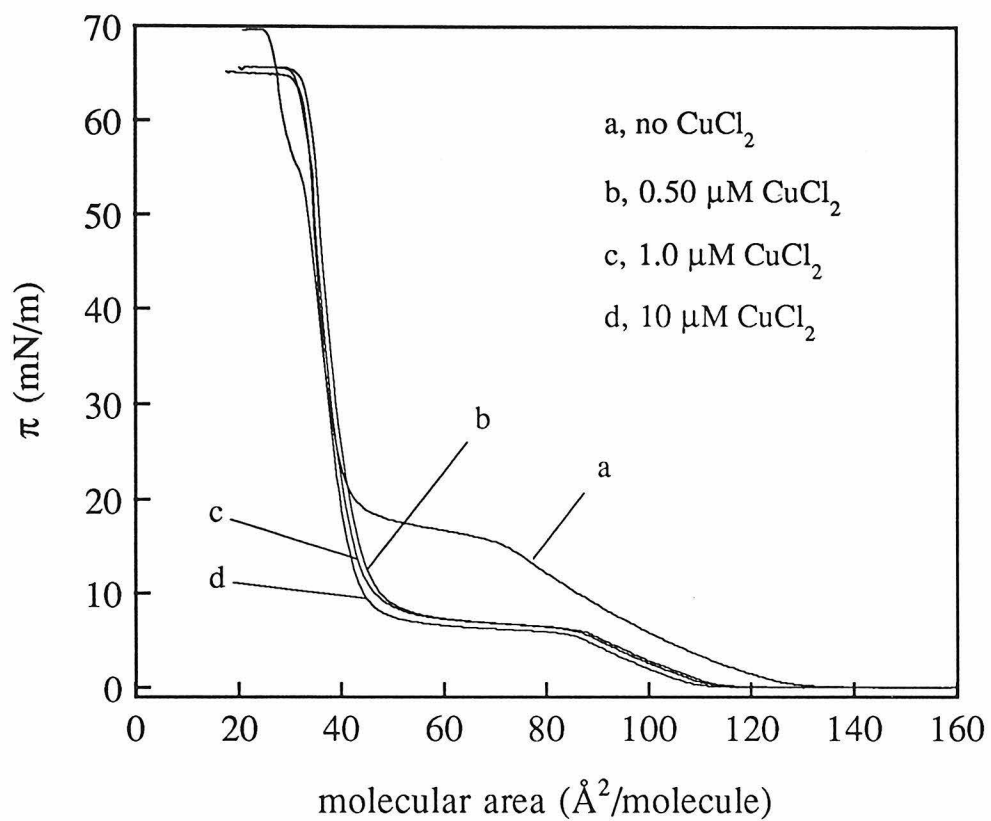


Figure 2.4. Surface pressure-molecular area isotherms of pure DPIDA lipid in the presence of varying amounts of CuCl_2 ; curve a, no divalent metal; b, $0.50 \mu\text{M}$ CuCl_2 ; c, $1.0 \mu\text{M}$ CuCl_2 and d, $10 \mu\text{M}$ CuCl_2 on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C).

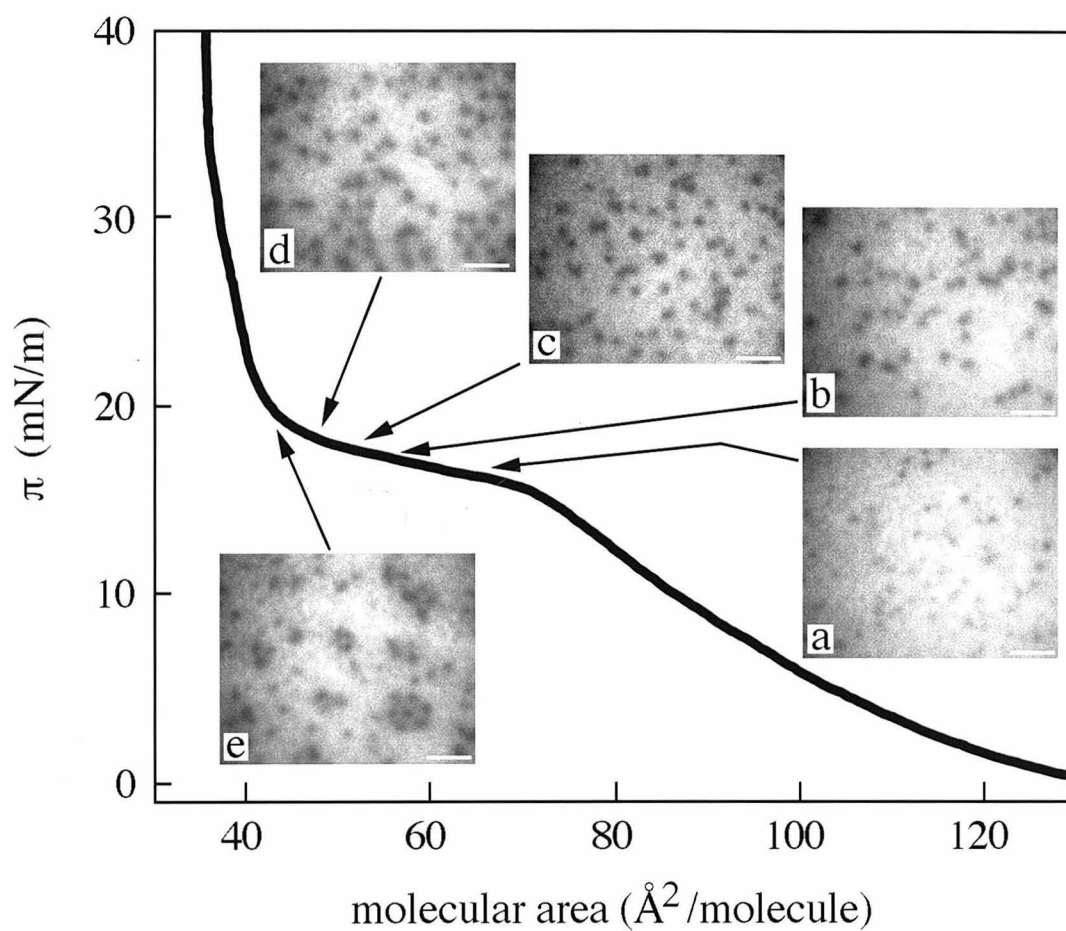


Figure 2.5a. Fluorescence micrographs of DPIDA monolayer (doped with 1 mol% NBD-PC) at various points throughout the phase transition (a, $\pi = 16.4$ mN/m; b, 17.2 mN/m; c, 17.6 mN/m; d, 18.5 mN/m; e, 19.1 mN/m). Subphase is 20 mM MOPS, 100 mM NaCl, pH 7.5 at room temperature. Scale bar in each image is 50 μm .

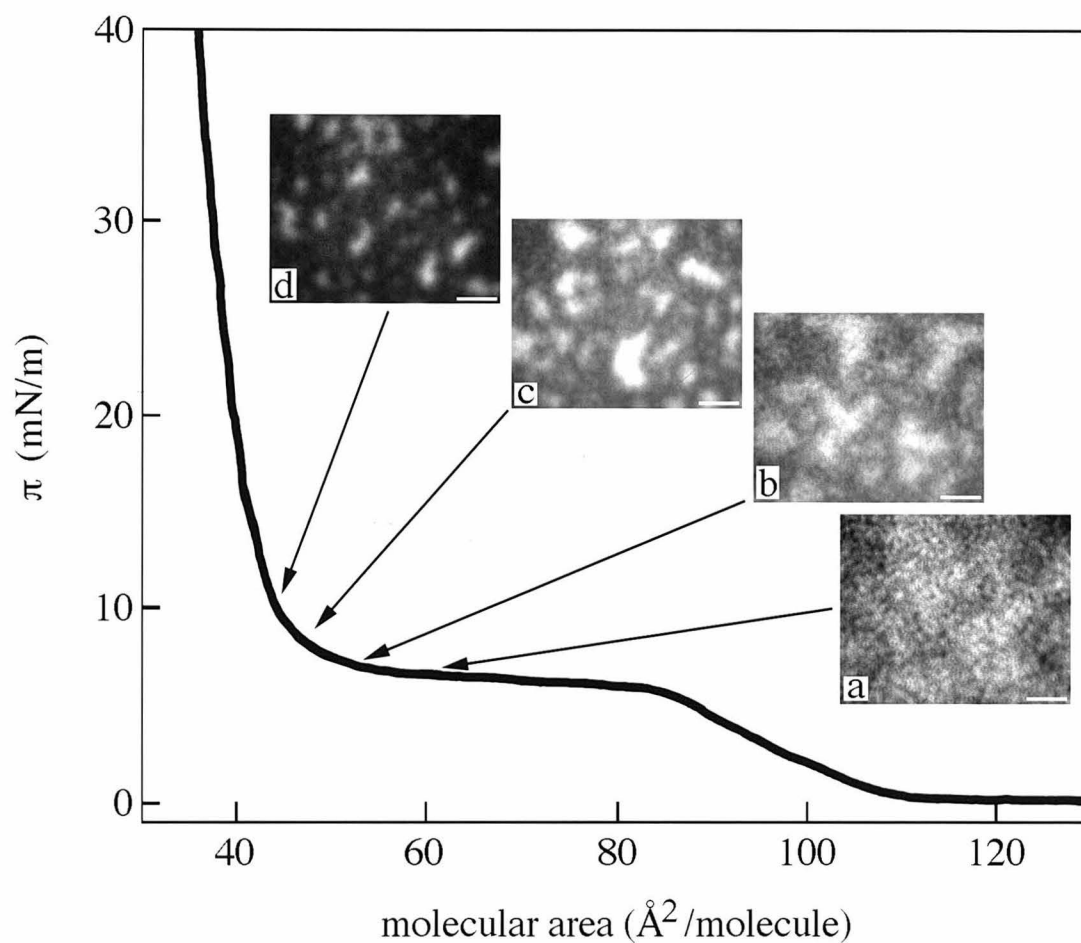


Figure 2.5b. Fluorescence micrographs of DPIDA monolayer (doped with 1 mol% NBD-PC) in the presence of copper at various points throughout the phase transition (a, $\pi = 6.5$ mN/m; b, 7.1 mN/m; c, 8.2 mN/m; d, 10.5 mN/m). Free Cu^{2+} in the subphase appears to quench the lipid fluorescence at low areas. Subphase is 20 mM MOPS, 100 mM NaCl, 10 mM CuCl_2 , pH 7.5 at room temperature. Scale bar in each image is 50 μm .

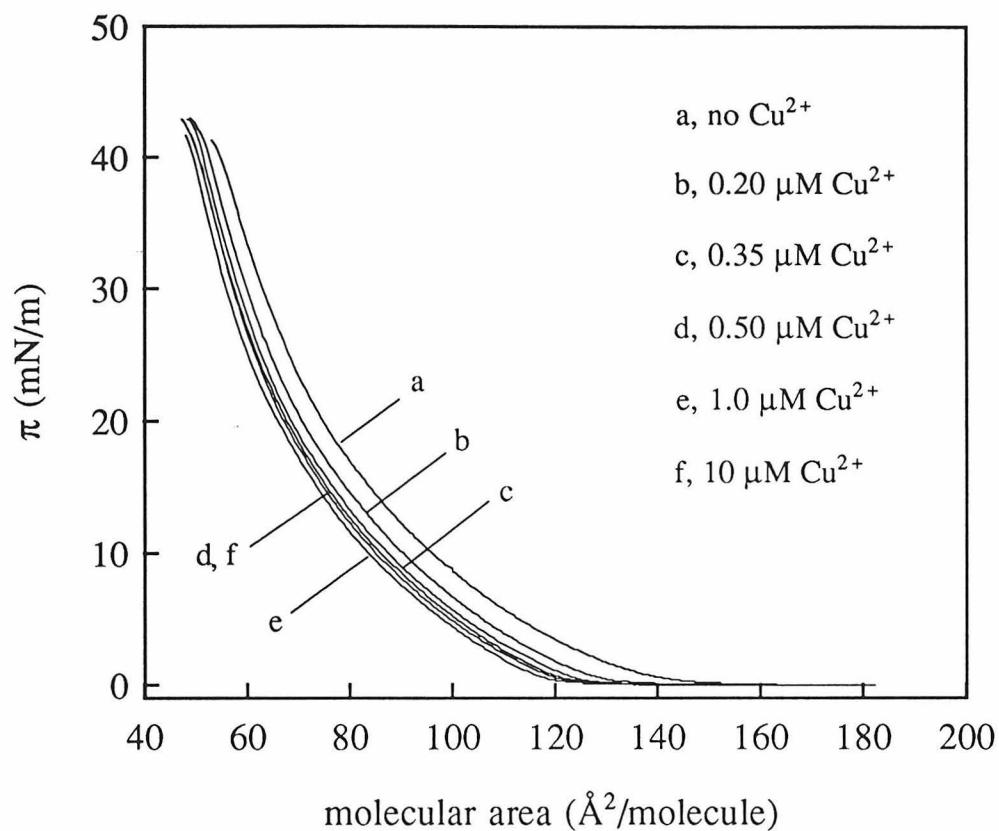


Figure 2.6. Surface pressure-molecular area isotherms of pure DOIDA lipid in the presence of varying amounts of CuCl_2 ; curve a, no divalent metal; b, $0.20 \mu\text{M CuCl}_2$; c, $0.35 \mu\text{M CuCl}_2$; d, $0.50 \mu\text{M CuCl}_2$; e, $1.0 \mu\text{M CuCl}_2$; and f, $10 \mu\text{M CuCl}_2$ on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25°C).

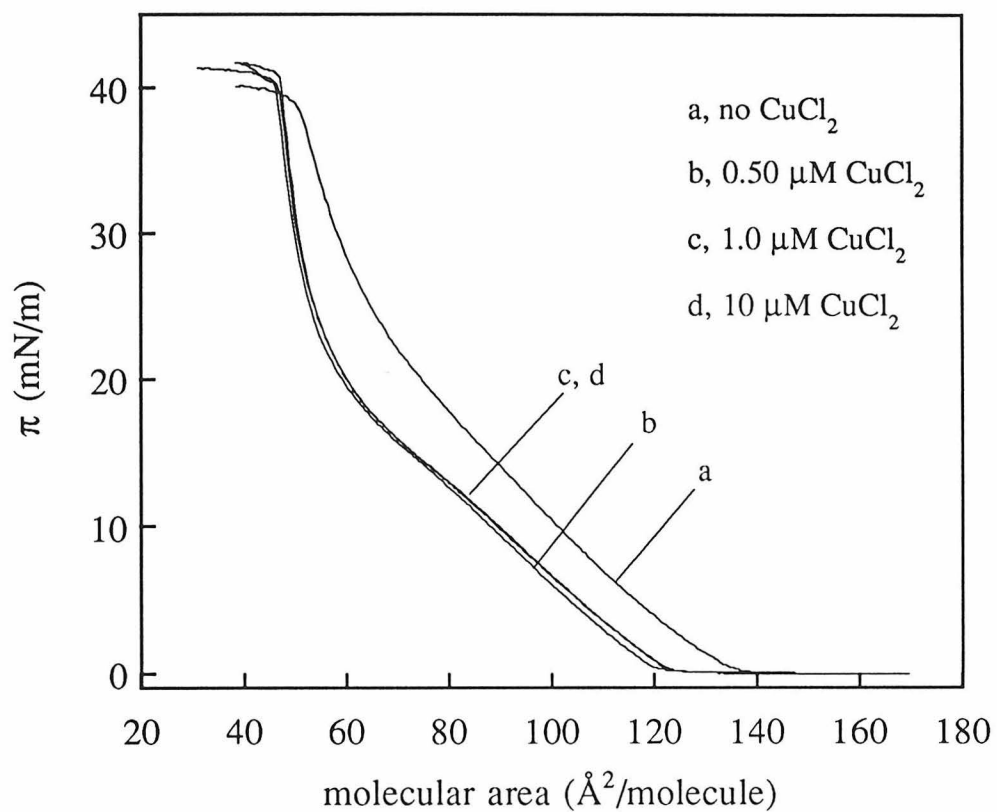


Figure 2.7. Surface pressure-molecular area isotherms of pure PSIDA lipid in the presence of varying amounts of CuCl_2 ; curve a, no divalent metal; b, $0.50 \mu\text{M}$ CuCl_2 ; c, $1.0 \mu\text{M}$ CuCl_2 ; and d, $10 \mu\text{M}$ CuCl_2 on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C).

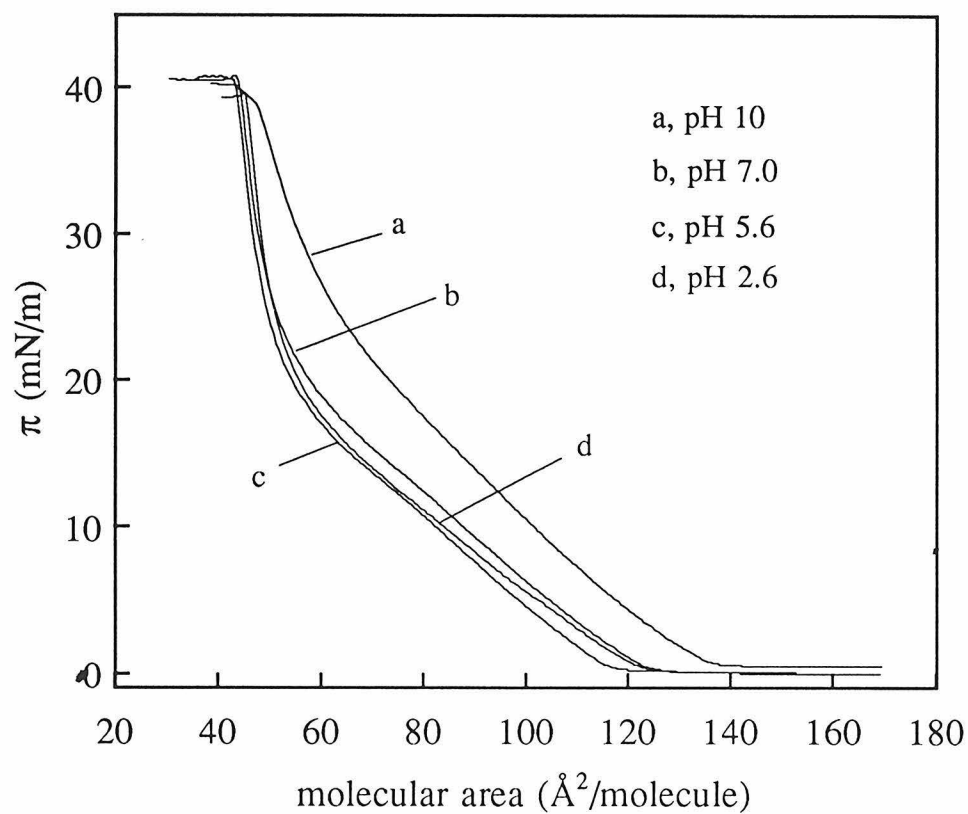


Figure 2.8. Surface pressure-molecular area isotherms of pure PSIDA lipid as a function of pH; curve a, pH 10; b, pH 7.0; c, pH 5.6; and d, pH 2.6. Suphase is pure water adjusted to appropriate pH with either HCl or NaOH. $T = 25\text{ }^{\circ}\text{C}$.

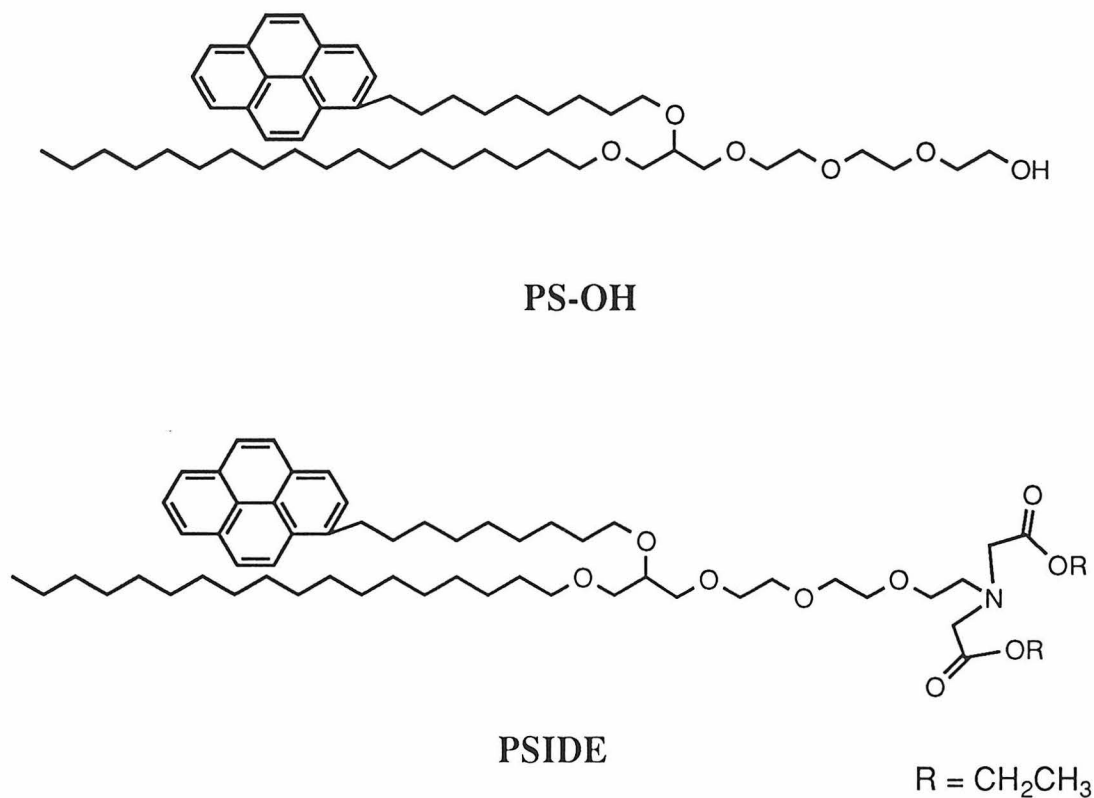


Figure 2.9. Structures of synthetic lipids 1-stearyl-2-(9-(1-pyrene)-nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-ol) (PS-OH) and 1-stearyl-2-(9-(1-pyrene)nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diethyldiacetate) (PSIDE). These lipids are identical to the metal chelating PSIDA lipid except for the headgroups. These lipids are not capable of chelating metal ions.

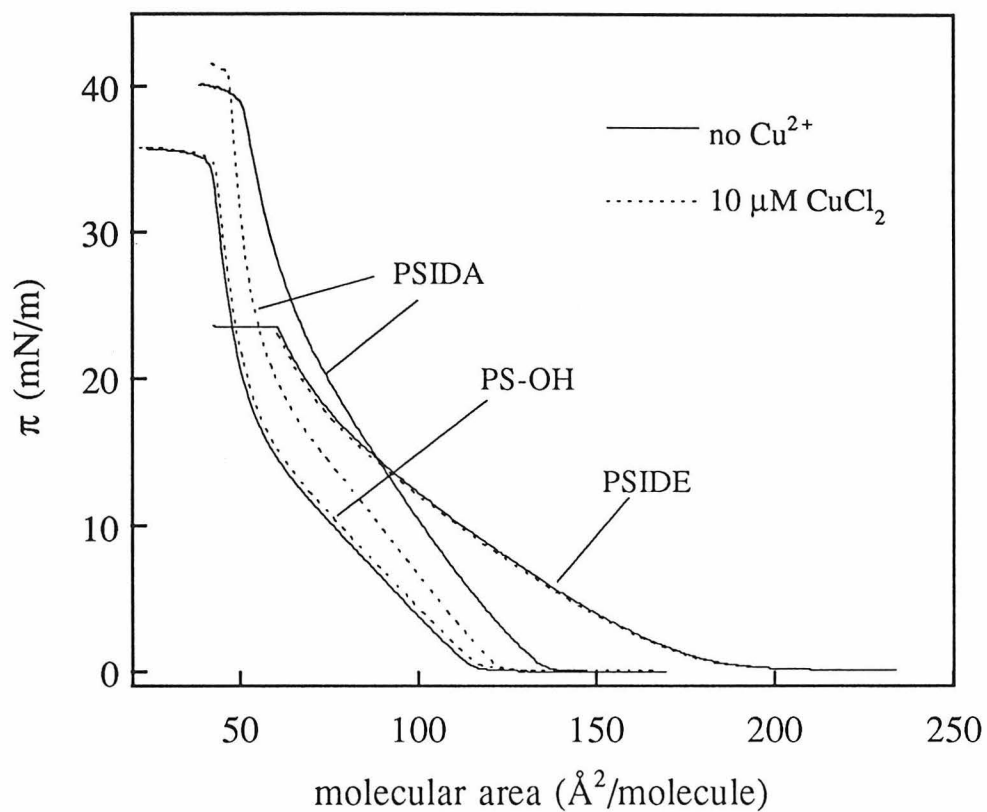


Figure 2.10. Surface pressure-molecular area isotherms of PSIDA and the synthetic intermediates PS-OH and PSIDE in the presence (10 μM CuCl_2) and absence of metal ion on buffered suphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 $^{\circ}\text{C}$).

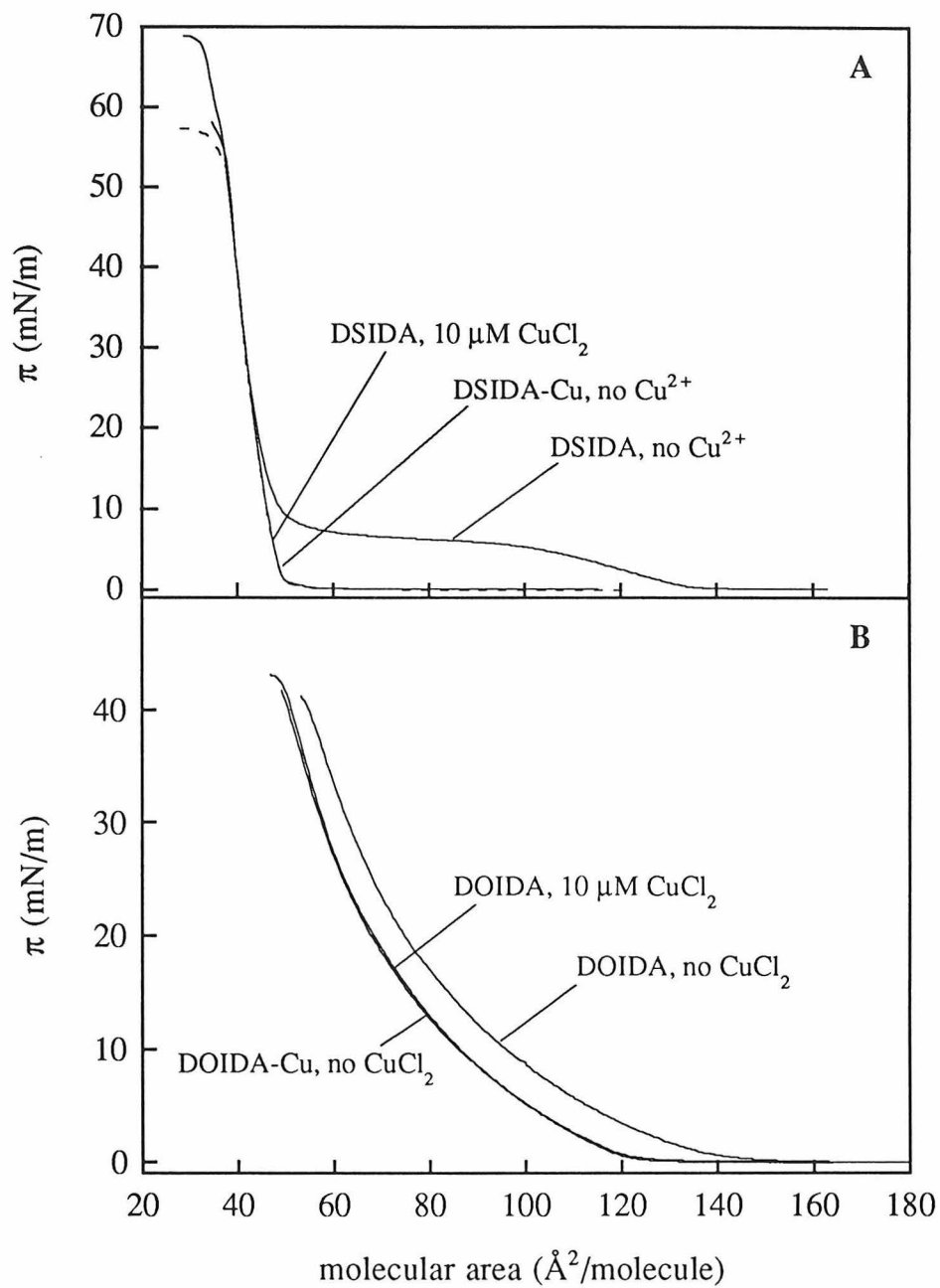


Figure 2.11. Surface pressure-molecular area isotherms of (A) DSIDA and (B) DOIDA showing the effect of pre-metallation of the lipids. Subphase is 20 mM MOPS, 100 mM NaCl, pH 7.5 plus CuCl_2 as indicated. $T = 25^\circ\text{C}$.

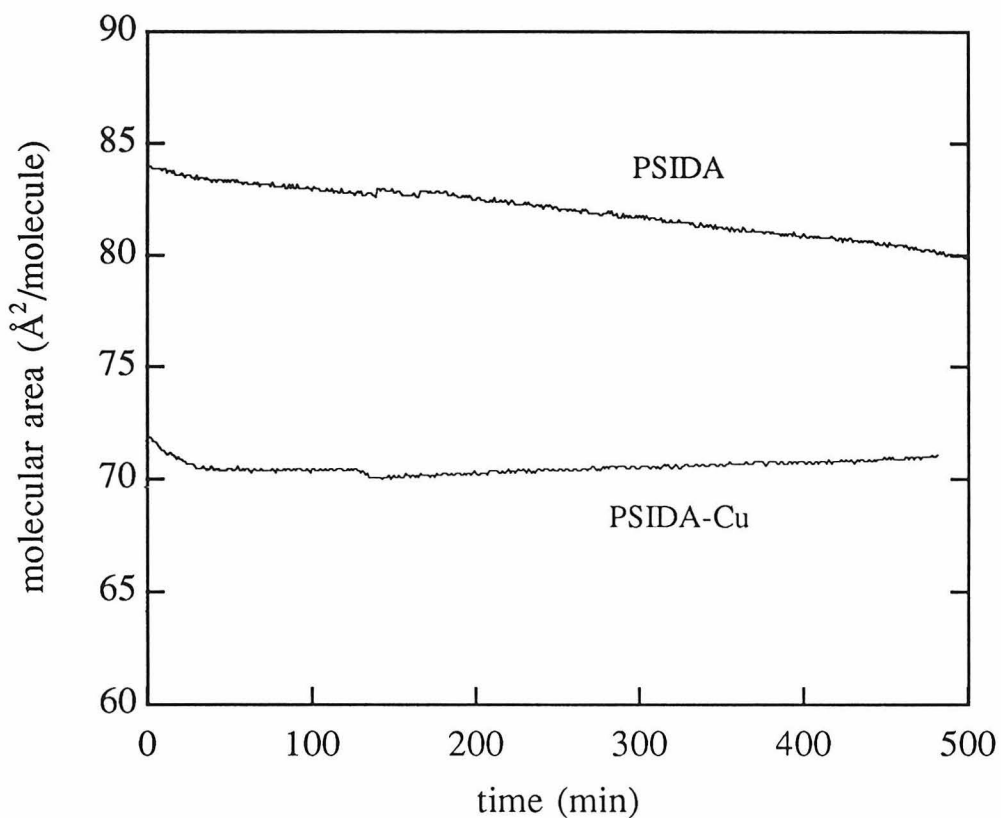


Figure 2.12. Molecular areas of PSIDA monolayers as a function of time. Top curve, un-metalated; bottom curve, pre-metalated lipid on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C). PSIDA decreases in area from 84 to 80 $\text{\AA}^2/\text{molecule}$. PSIDA-Cu increases in area slightly from 70 (at $t = 20$ min) to 71 $\text{\AA}^2/\text{molecule}$.

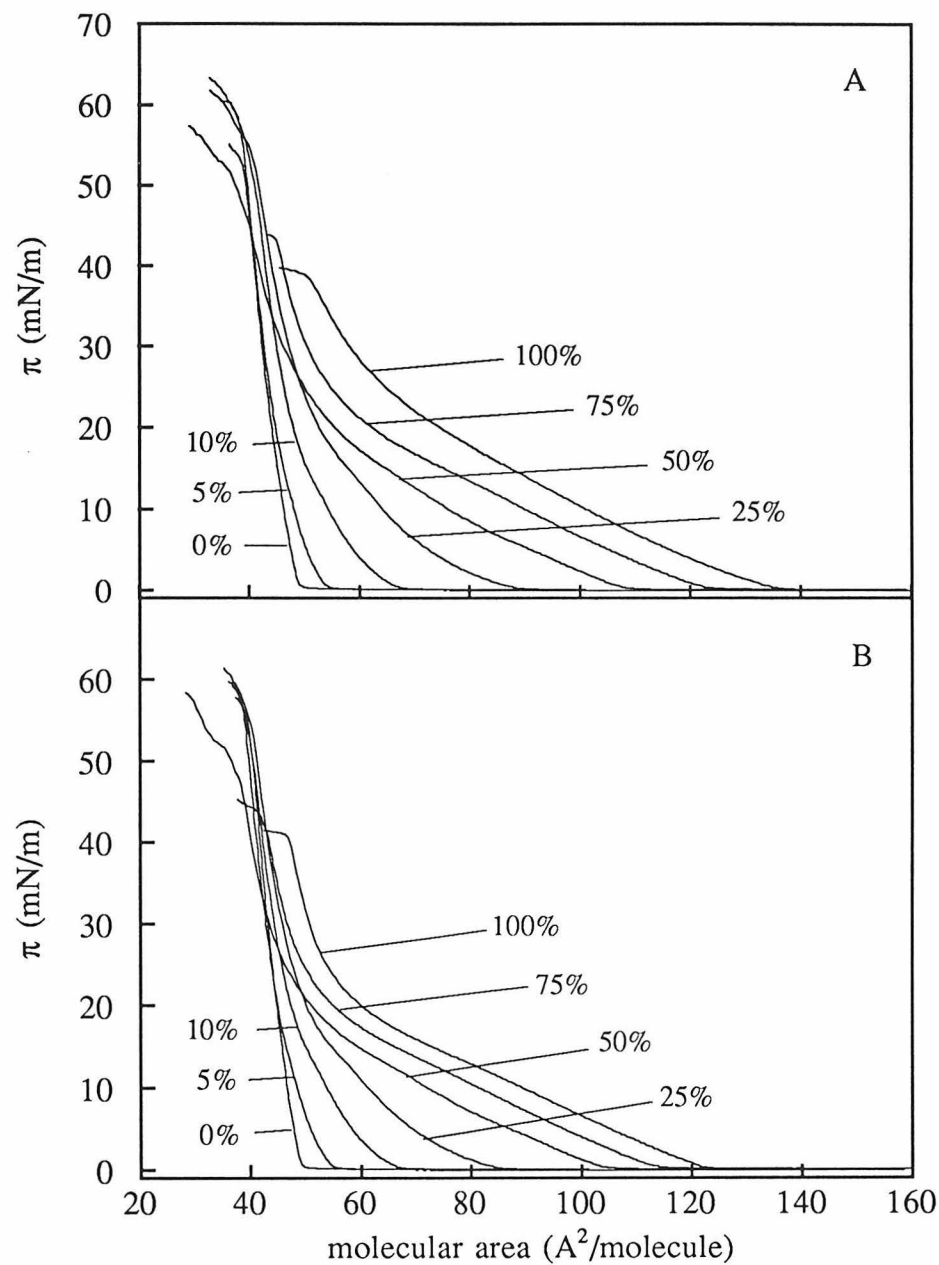


Figure 2.13. Surface pressure-molecular area isotherms of PSIDA/DSPC mixed monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5). (A) no divalent metal ion; (B) 10 μM CuCl_2 in subphase. Isotherms are labeled as mol% PSIDA lipid. $T = 25^\circ\text{C}$.

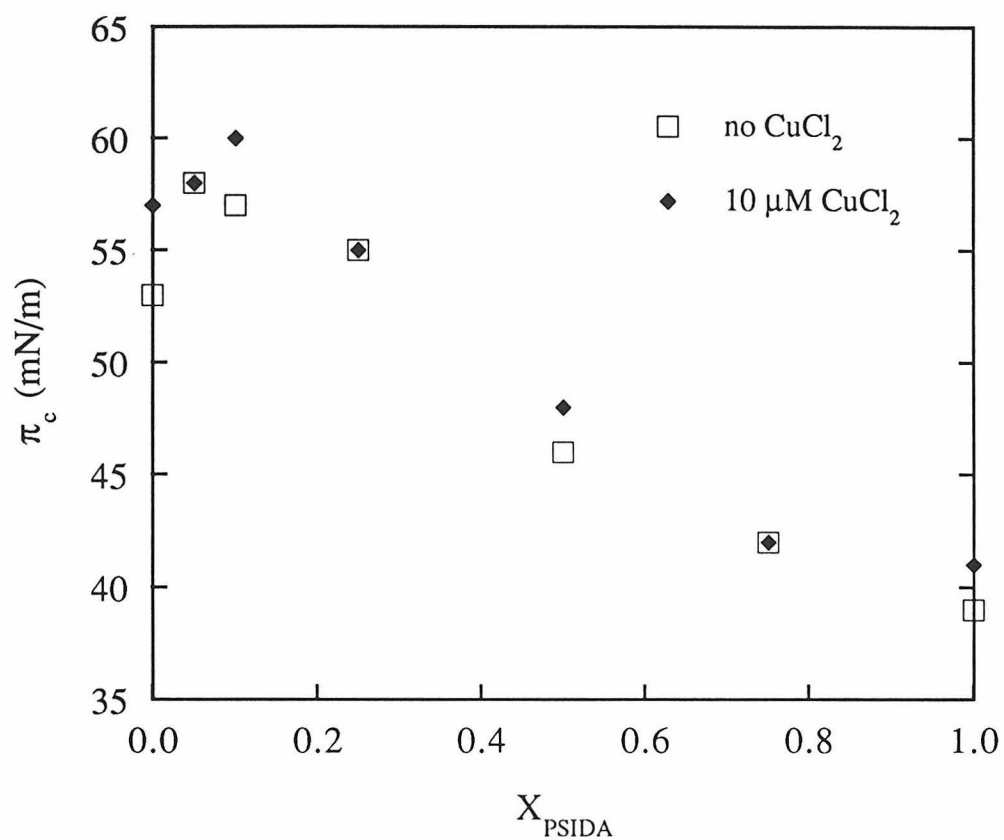


Figure 2.14. Collapse pressures vs. PSIDA mole fraction for PSIDA/DSPC mixed monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5) in the absence (open symbols) and presence (filled symbols) of 10 μM CuCl_2 .

Experimental error is approximately the size of the symbols. $T = 25^\circ\text{C}$.

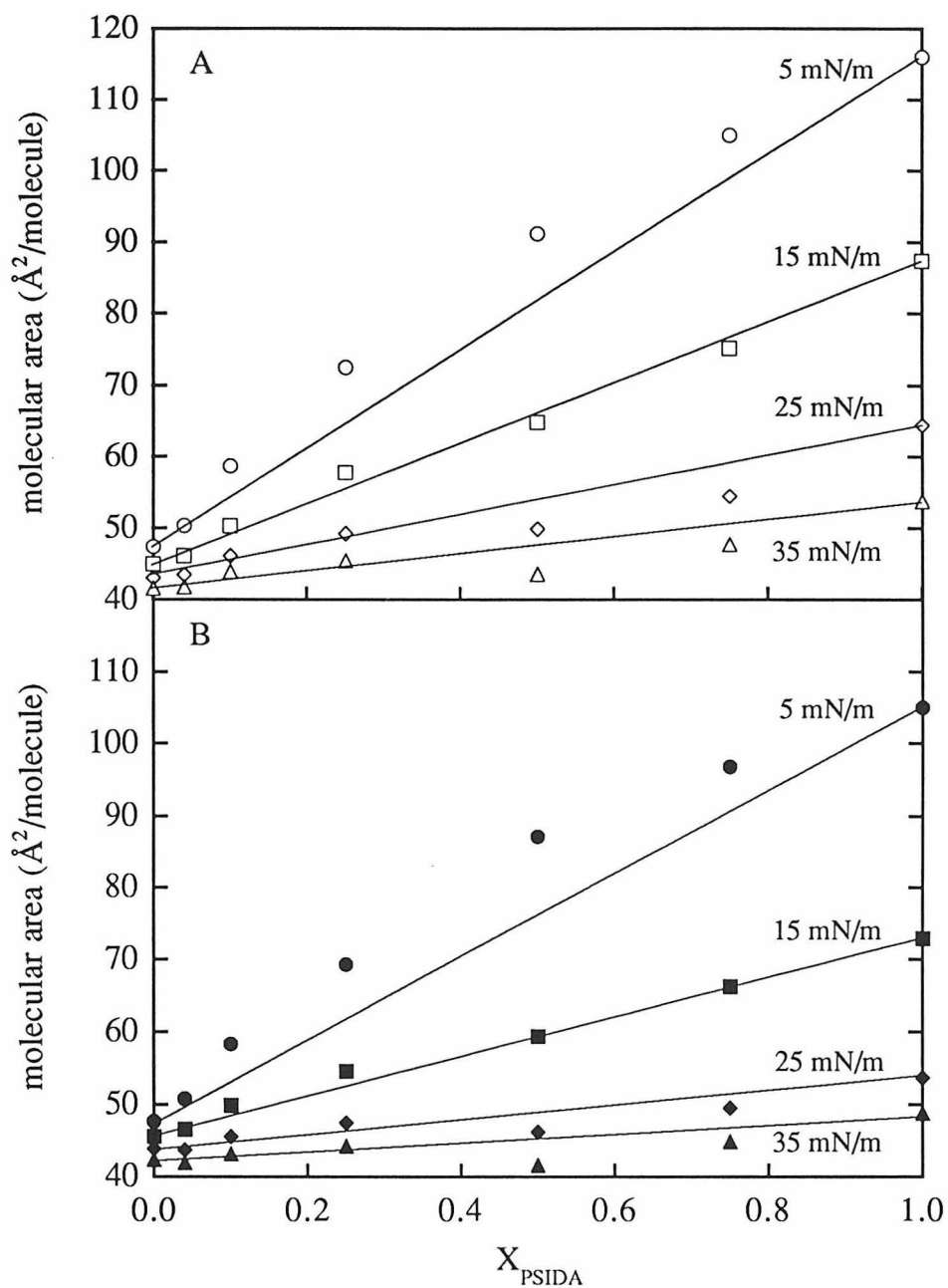


Figure 2.15. Mean molecular area plots for PSIDA/DSPC mixed monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C) in the absence (A) and presence (B) of 10 $\mu\text{M CuCl}_2$ at various surface pressures as indicated.

Solid line represents “ideal” behavior. Data taken from Fig. 2.13. Experimental error in molecular areas is approximately the size of the symbols.

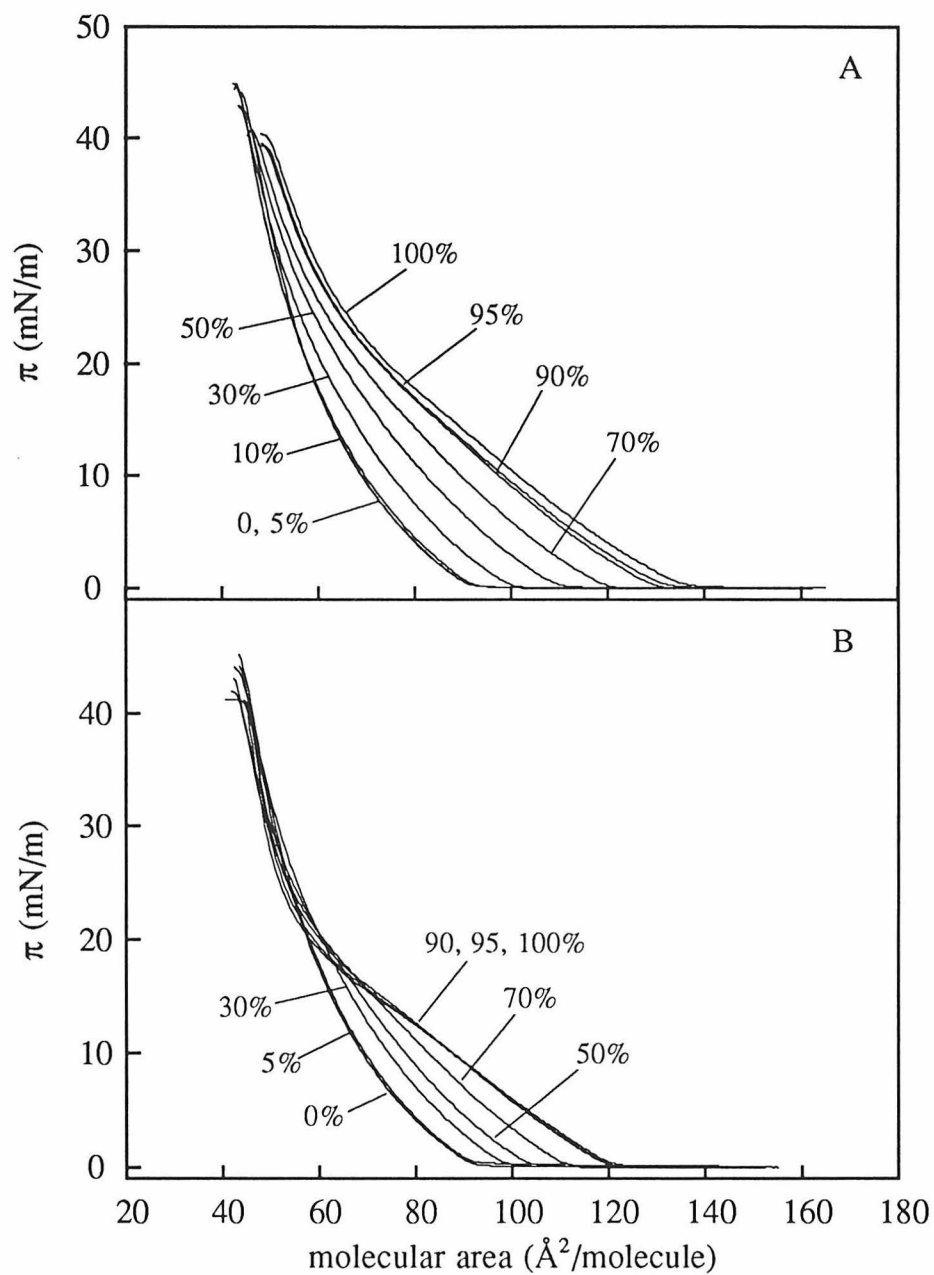


Figure 2.16. Surface pressure-molecular area isotherms of PSIDA/SOPC mixed monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5). (A) no divalent metal ion; (B) 10 μM CuCl_2 in subphase. Isotherms are labeled as mol% PSIDA lipid. $T = 25^\circ\text{C}$.

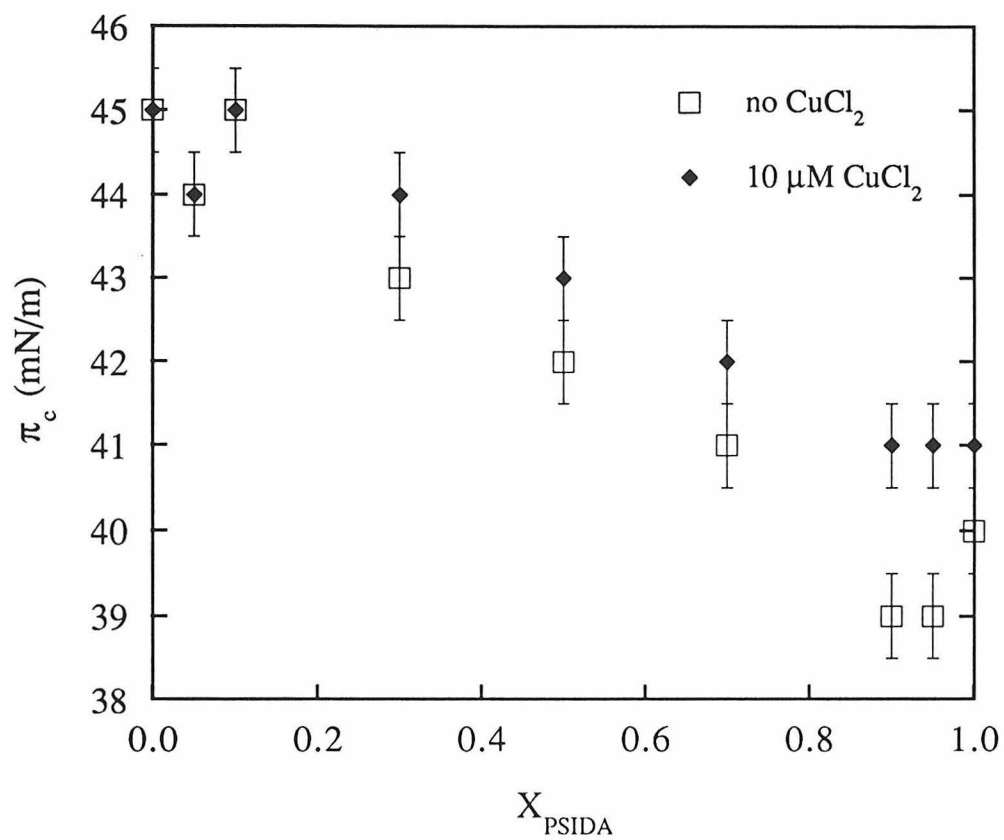


Figure 2.17. Collapse pressure vs. PSIDA mole fraction for PSIDA/SOPC mixed monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5) in the absence (open symbols) and presence (filled symbols) of 10 μM CuCl_2 .

T = 25 °C.

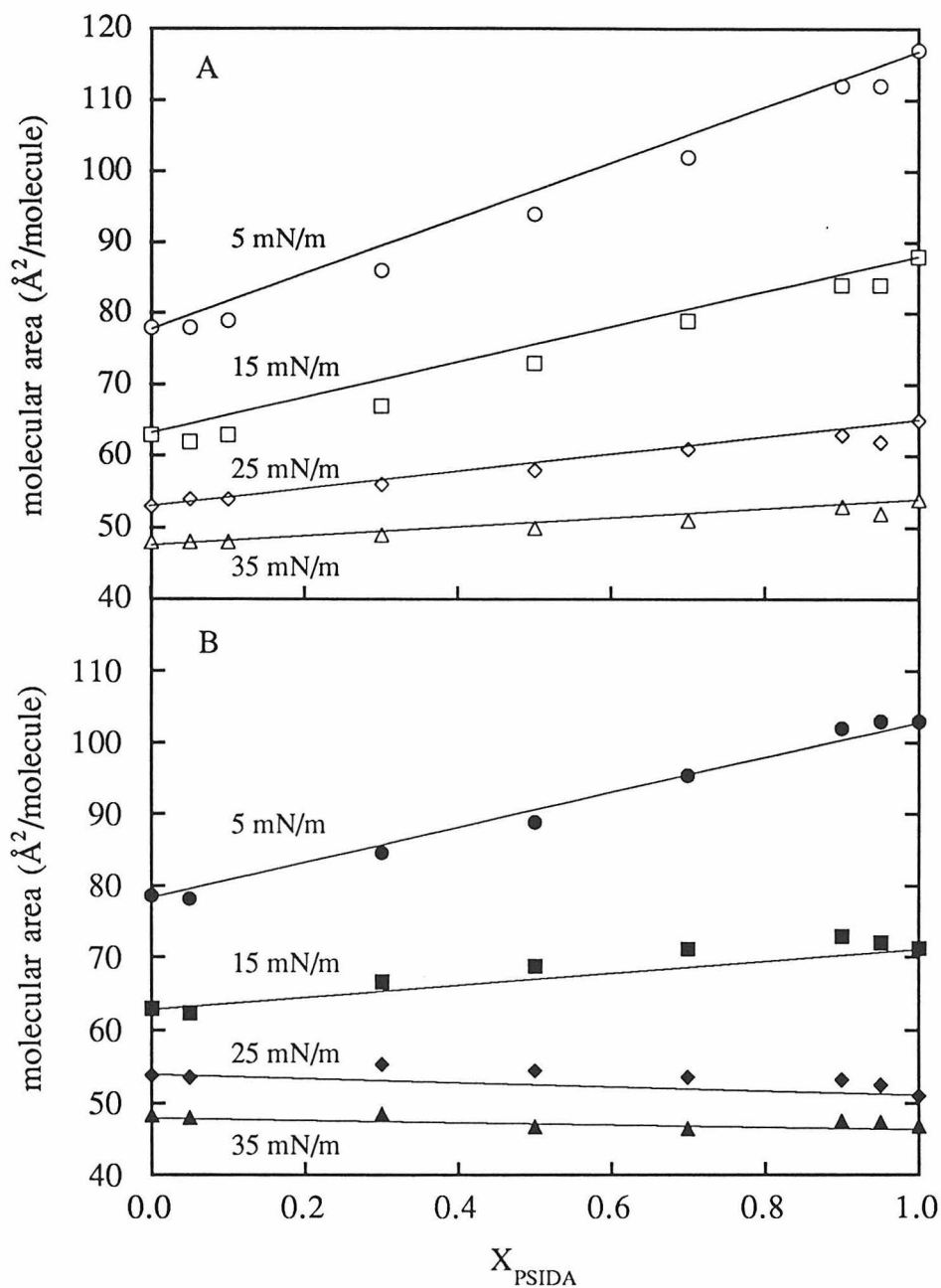


Figure 2.18. Mean molecular area plots for PSIDA/SOPC mixed monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C) in the absence (A) and presence (B) of $10 \mu\text{M CuCl}_2$ at various surface pressures as indicated. Solid line represents “ideal” behavior. Data taken from Fig. 2.16. Experimental error in molecular areas is approximately the size of the symbols.

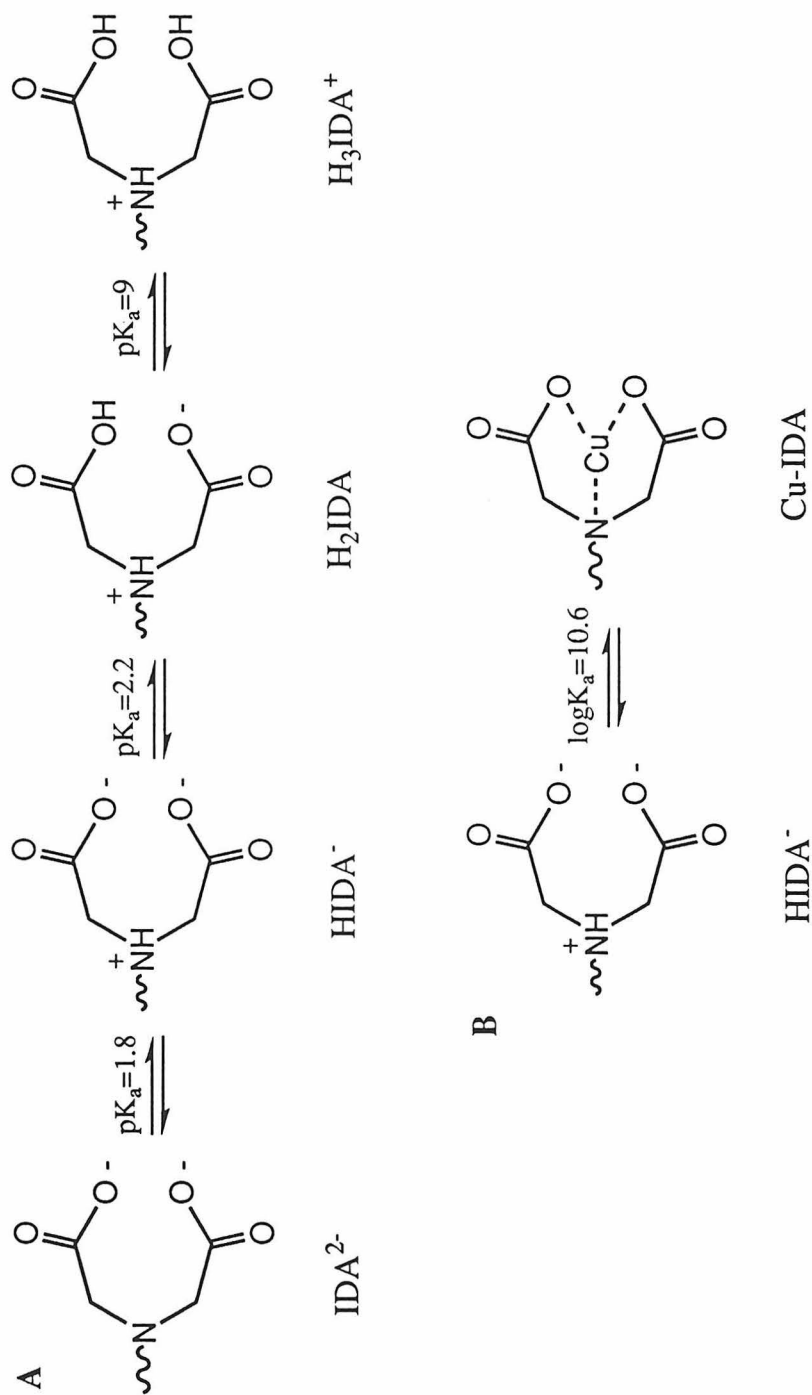


Figure 2.19. Schematic showing (A) the possible protonation states of the IDA headgroups and their overall charge and (B) the expected protonation states of the unmetalated and metalated IDA headgroups for lipid on buffered subphase at pH 7.5. The association constant given is for IDA and Cu²⁺ in solution; binding at the interface is somewhat weaker.

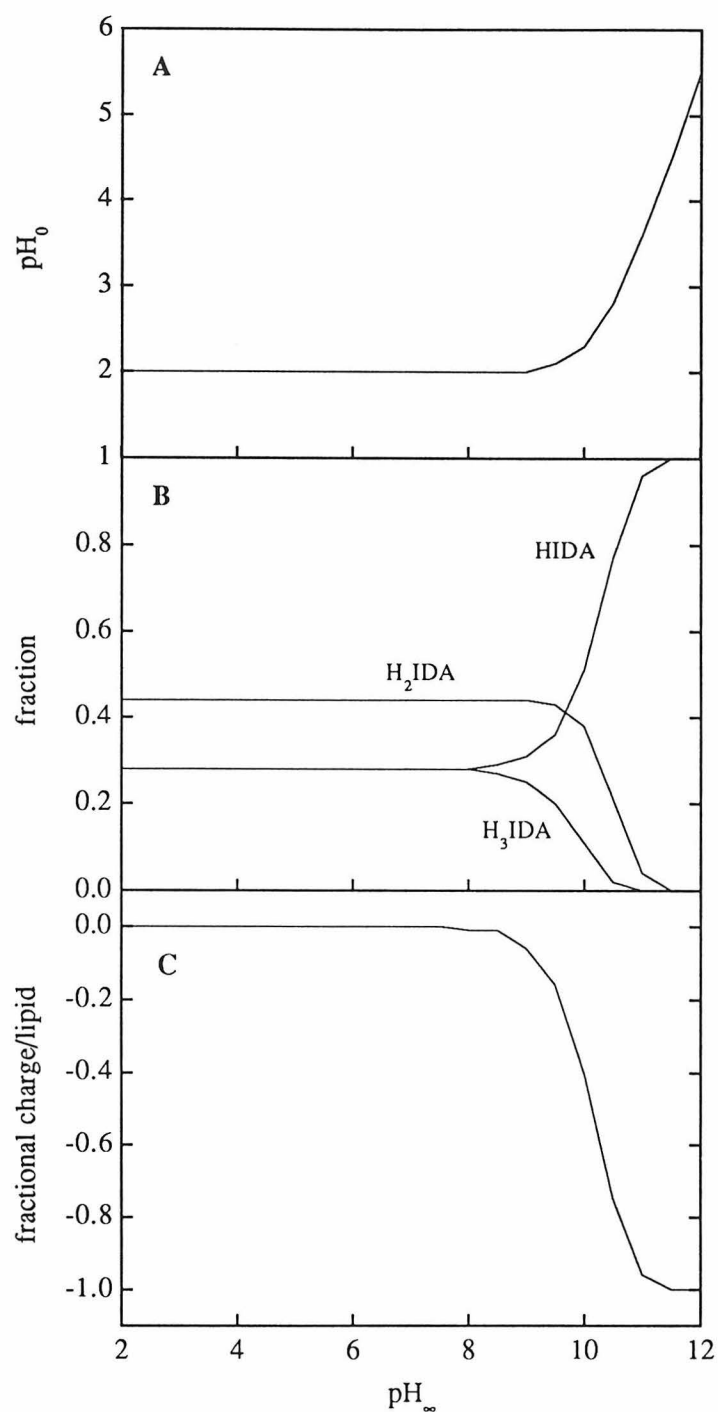


Figure 2.20. (A) Subphase pH at the interface, (B) species distributions, and (C) fractional charge per lipid at varying subphase pH as predicted by the Gouy-Chapman-Stern theory, equations (1) and (4), for an IDA lipid at the air-water interface. Lipid area was chosen to be $50 \text{ \AA}^2/\text{molecule}$, $T = 25 \text{ }^\circ\text{C}$.

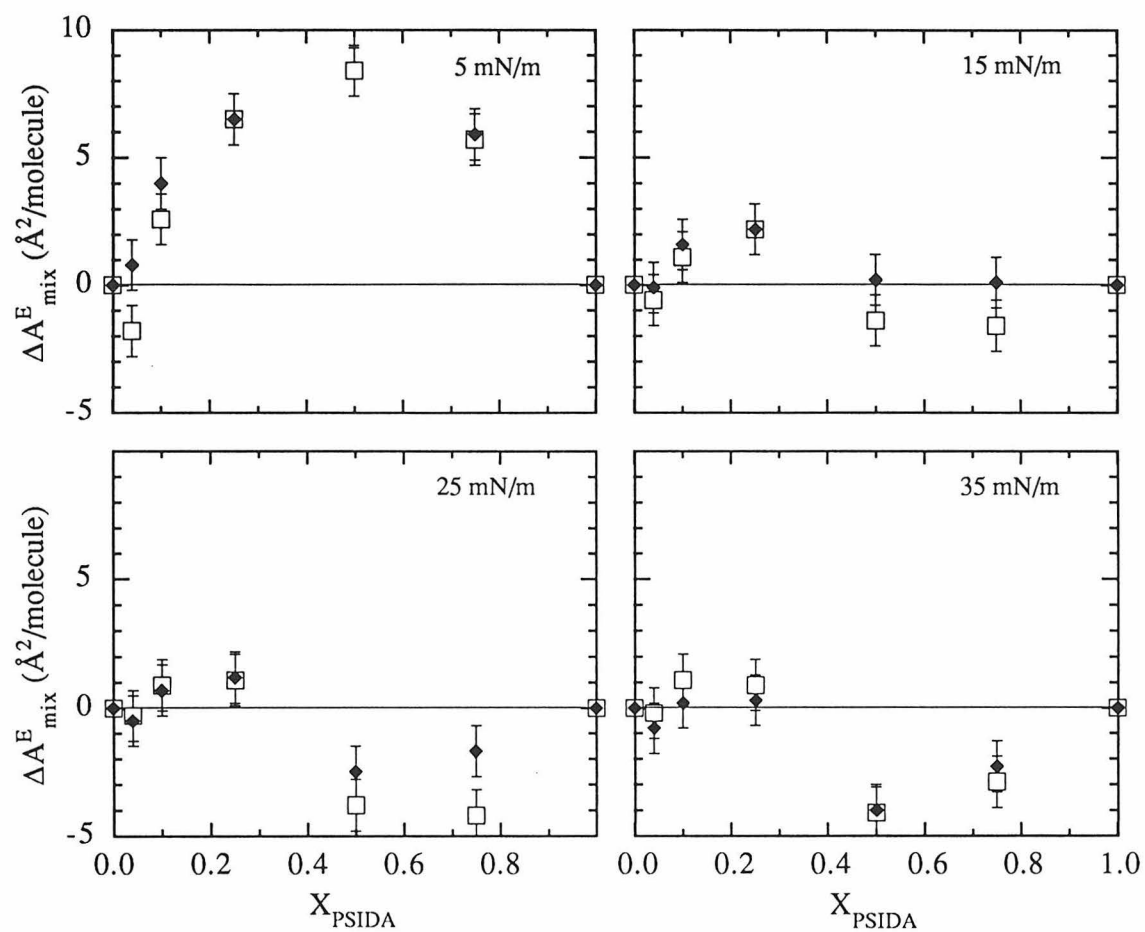


Figure 2.21. Excess areas of mixing for PSIDA/DSPC mixed monolayers as a function of mole fraction PSIDA ($\square$, no metal; $\blacklozenge$, $10 \mu\text{M CuCl}_2$). Excess areas were calculated according to equation (1) using data from Figure 2.13.

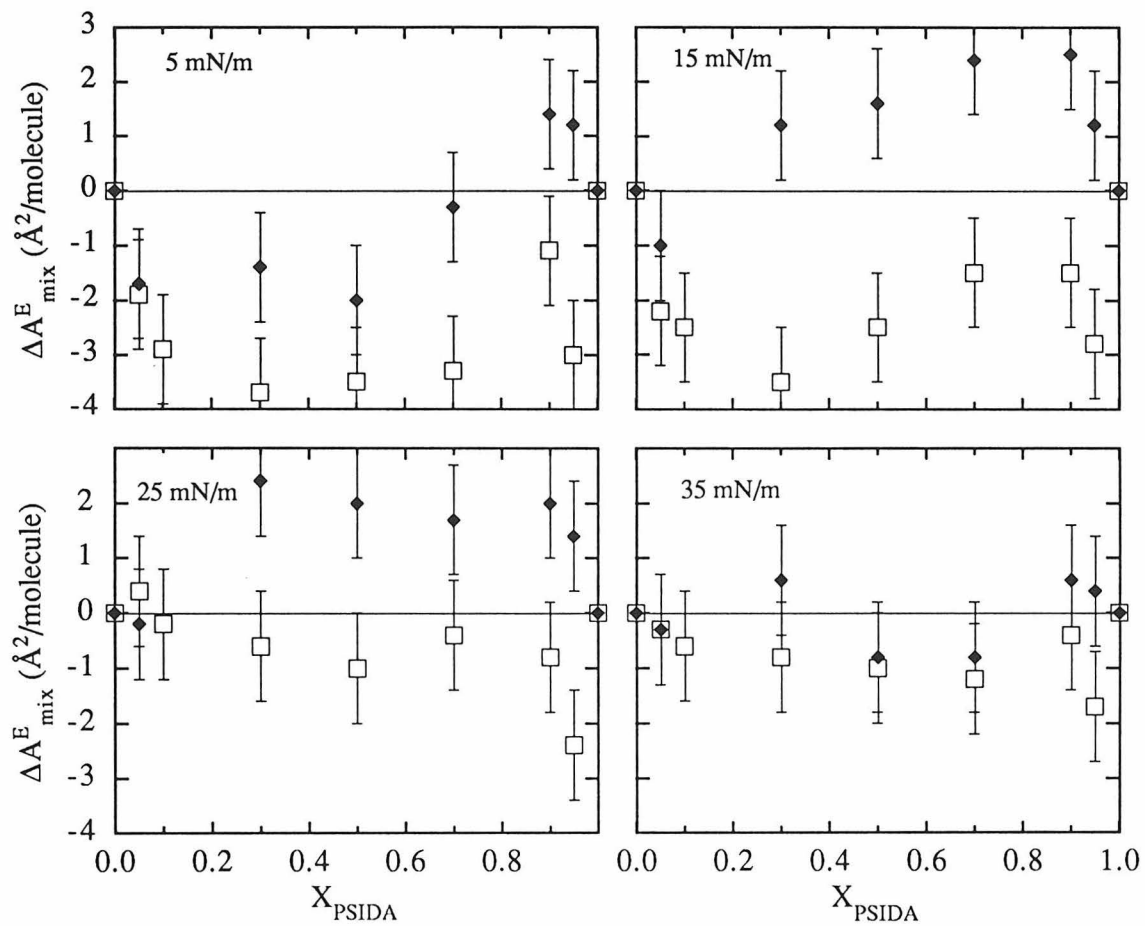


Figure 2.22. Excess areas of mixing for PSIDA/SOPC mixed monolayers as a function of mole fraction PSIDA ($\square$, no metal; $\blacklozenge$, $10 \mu\text{M CuCl}_2$). Excess areas were calculated according to equation (1) using data from Figure 2.16.

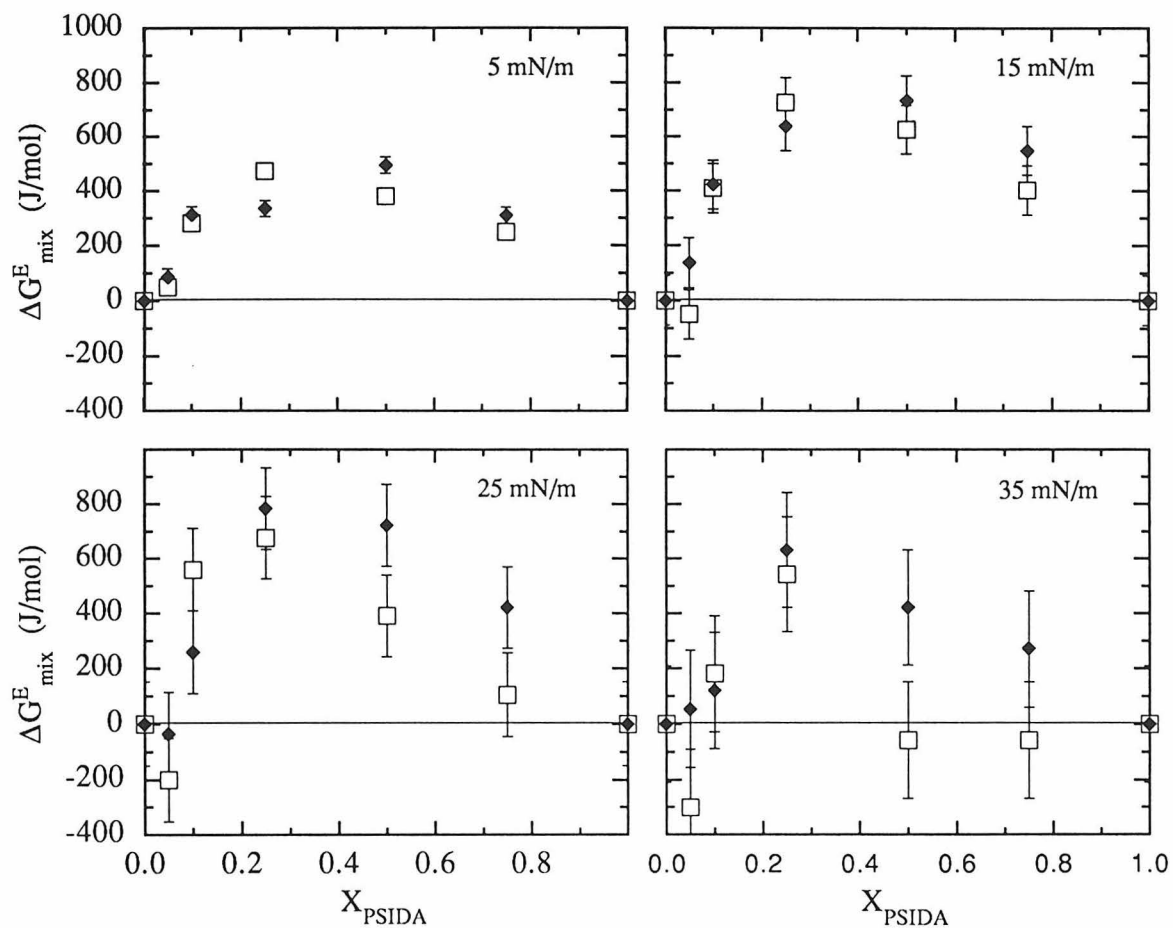


Figure 2.23. Excess free energies of mixing as a function of PSIDA mole fraction for PSIDA/DSPC mixed monolayers at varying surface pressure as indicated ($\square$, no metal; $\blacklozenge$, $10 \mu\text{M CuCl}_2$). Free energies were calculated according to equation (10). Error (J/mol) is approximately six times the surface pressure as shown.

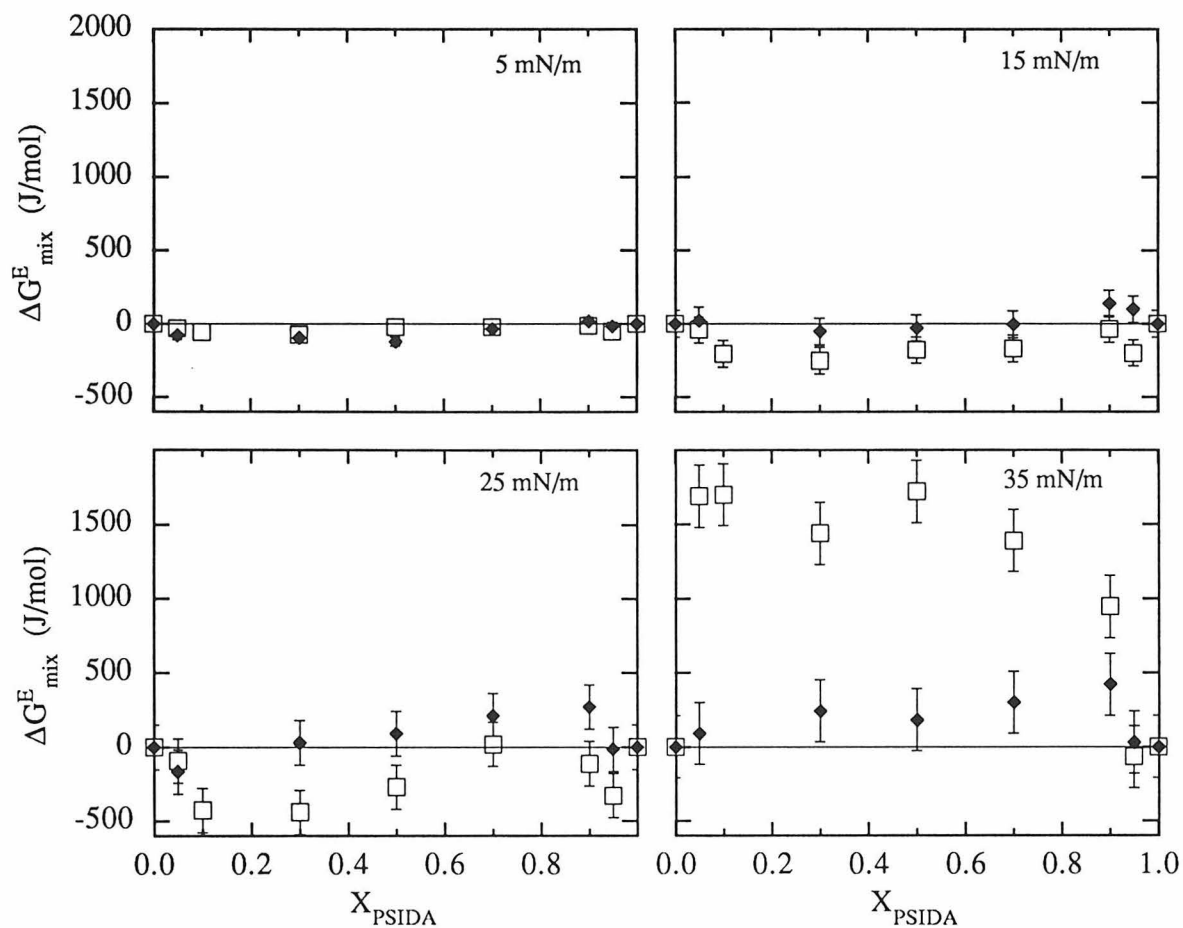


Figure 2.24. Excess free energies of mixing as a function of PSIDA mole fraction for PSIDA/SOPC mixed monolayers at varying surface pressure as indicated ($\square$, no metal; $\blacklozenge$, 10 μM CuCl_2). Free energies were calculated according to equation (10). Error (J/mol) is approximately six times the surface pressure as shown.

Appendix. Gouy-Chapman-Stern Theory

A monolayer of ionizable lipids at the air-water interface represents a charged surface with a relatively large charge density. The charge on the surface must, of course, be neutralized by counterions in the phase near the interface. In general, the charged surface produces an electric field that attracts counterions and repels coions such that near the interface the solution is enriched in counterions and depleted in coions relative to the bulk aqueous solution. This region of altered ion distribution is known as the diffuse double layer [50], and the theories of Gouy [65] and Chapman [66] seek to describe it. Many excellent reviews have covered this subject (c.f. [50] and references therein) and it will be described here in concept only.

The Poisson-Boltzmann equation, which balances the attraction (or repulsion) of ions by the electric field at the interface with the statistical diffusion of the ions away from (or toward) the interface, can be solved with the appropriate boundary conditions to give an equation for the potential as a function of distance from the interface. The potential decays exponentially with distance from the surface, falling to $1/e$ its value at the surface at a distance $1/\kappa$, the Debye length. The Debye length decreases with increasing ionic strength in the aqueous phase. By invoking the restriction of electroneutrality in the bulk phase, the following relation between the potential in the aqueous phase at the interface, Ψ_0 , and the surface charge density, σ , is obtained:

$$\sigma = (8\epsilon_r\epsilon_0[c]_{\infty}NkT)^{1/2} \sinh(ze\Psi_0/2kT) \quad (\text{A.1})$$

where ϵ_r and ϵ_0 are the dielectric constants of water and vacuum, respectively; $[c]_{\infty}$ is the bulk ion concentration; N is Avogadro's number; k is the Boltzmann constant; T is temperature; and e is electronic charge. It should be noted that this derivation assumes (1)

the ions are point charges, (2) the dielectric constant is equal to its bulk value up to the interface, (3) image charge effects, ion-dipole forces which tend to attract the ions back into the high dielectric bulk phase as they approach the low dielectric membrane, are negligible and (4) the surface charge is non-discrete or “smeared” over the entire surface of the membrane, none of which are strictly true. Nonetheless, the Gouy-Chapman theory is a valuable tool for investigating phenomena which depend on the average value of the potential as a function of distance from the membrane, such as local values of pH and ion concentrations. In fact, much experimental evidence has shown this theory provides a remarkably good description of the electrostatic potential due to charged, artificial membranes. The above equation was derived for a single, symmetric electrolyte in solution. By following the same derivation considering other electrolytes, one arrives at the more general Grahame equation (1)

$$\sigma = - \left[2\epsilon_r \epsilon_0 kT \sum_i [c_i]_{\infty} \left\{ \exp \left(- \frac{z_i e \Psi_0}{kT} \right) - 1 \right\} \right]^{1/2} \quad (\text{A.2})$$

where $[c_i]_{\infty}$ and z_i are the bulk concentration and valence of ionic species i , respectively, and the summation is over all ionic species in solution.

In the case where ions in solution bind to the lipids, we must consider the effect of their adsorption on the membrane surface charge density. We can utilize the Langmuir adsorption isotherm to describe the states of the surface binding sites (lipids) as a function of ion concentration in the aqueous phase. For the case of IDA lipids, we have three equilibria

$$[H_3IDA^+] = K_{a3} [H_2IDA] [H^+]_0 = K_{a1} K_{a2} K_{a3} [IDA^{2-}] [H^+]_0^3 \quad (A.3)$$

$$[H_2IDA] = K_{a2} [HIDA^-] [H^+]_0 = K_{a1} K_{a2} [IDA^{2-}] [H^+]_0^2 \quad (A.4)$$

$$[HIDA^-] = K_{a1} [IDA^{2-}] [H^+]_0 \quad (A.5)$$

and $[H^+]_0$ is the interfacial proton concentration. The protonation constant of the IDA lipid headgroups at the interface are not known and, for the present analysis, are assumed to be equal to those for IDA in bulk solution [32]. The surface charge density is given by

$$\sigma = e (2[IDA^{2-}] + [HIDA^-] - [H_3IDA^+]) \quad (A.6)$$

Combining A.6 with A.3, A.4 and A.5, we get the surface charge density in terms of one species, $[IDA^{2-}]$ as follows:

$$\sigma = e \{ [IDA^{2-}] (2 + K_{a1} [H^+]_0 - K_{a1} K_{a2} K_{a3} [H^+]_0^3) \} \quad (A.7)$$

However, in general, only the total IDA concentration

$$[IDA]_{tot} = [IDA^{2-}] + [HIDA^-] + [H_2IDA] + [H_3IDA^+] \quad (A.8)$$

is known and is equivalent to the reciprocal of the experimentally accessible molecular area, A ($[IDA]_{tot} = 1/A$). Combining equation A.8 with equations A.3 - A.5, solving for $[IDA^{2-}]$ and substituting into equation A.7 the surface charge density becomes

$$\sigma = \left(\frac{e}{A} \right) \frac{2 + K_{a1} [H^+]_0 - K_{a1} K_{a2} K_{a3} [H^+]_0^3}{1 + K_{a1} [H^+]_0 + K_{a1} K_{a2} [H^+]_0^2 + K_{a1} K_{a2} K_{a3} [H^+]_0^3}. \quad (A.9)$$

The proton concentration in the aqueous phase adjacent to the surface is generally not known, but can be related to the bulk concentration through Boltzmann's equation (2)

$$[H^+]_0 = [H^+]_\infty \exp\left(-\frac{e\Psi_0}{kT}\right) \quad (A.10)$$

where $[H^+]_\infty$ is the known bulk proton concentration. Substituting equation A.10 into A.9 leads to the equation (4) for the surface charge density

$$\sigma = \left(\frac{e}{A}\right) \frac{2 + K_{a1}[H^+]_\infty \Phi - K_{a1}K_{a2}K_{a3}[H^+]_\infty^3 \Phi^3}{1 + K_{a1}[H^+]_\infty \Phi + K_{a1}K_{a2}[H^+]_\infty^2 \Phi^2 + K_{a1}K_{a2}K_{a3}[H^+]_\infty^3 \Phi^3} \quad (A.11)$$

where $\Phi = \exp(-e\Psi_0/kT)$. Equations A.2 and A.11 now provide two independent relations for the surface charge density, which can be solved iteratively for the surface potential, Ψ_0 . Once the potential is known, the interfacial proton concentration and IDA species distributions are easily determined.

The same analysis can be easily extended to describe the distribution of IDA lipid species on a buffered subphase in the presence of NaCl and CuCl₂ as follows. The affinities of the IDA headgroups for Na⁺ and Cu²⁺ require the addition of two expressions to the Langmuir adsorption isotherm, represented by

$$[IDA \cdot Cu] = K_{Cu} [Cu^{2+}]_0 [IDA^{2-}] \quad (A.12)$$

$$[IDA \cdot Na] = K_{Na} [Na^+]_0 [IDA^{2-}] \quad (A.13)$$

where K_{Cu} and K_{Na} are the interfacial association constants for IDA with Cu²⁺ and Na⁺, respectively; $[Cu^{2+}]_0$ is the concentration of Cu²⁺ at the interface and $[Na^+]_0$ is the concentration of Na⁺ at the interface. The balance on IDA then becomes

$$[IDA]_{tot} = [IDA^{2-}] + [HIDA^-] + [H_2IDA] + [H_3IDA^+] + [IDA \cdot Cu] + [IDA \cdot Na] \quad (A.14)$$

and finally

$$\sigma = \frac{\left(\frac{e}{A}\right)\left(2 + \left(K_{a1}[H^+]_{\infty} + K_{Na}[Na^+]_{\infty}\right)\Phi - K_{a1}K_{a2}K_{a3}[H^+]_{\infty}^3\Phi^3\right)}{1 + \left(K_{a1}[H^+]_{\infty} + K_{Na}[Na^+]_{\infty}\right)\Phi + \left(K_{a1}K_{a2}[H^+]_{\infty}^2 + K_{Cu}[Cu^{2+}]_{\infty}\right)\Phi^2 + K_{a1}K_{a2}K_{a3}[H^+]_{\infty}^3\Phi^3}$$

(A.15)

Again, this equation can be solved simultaneously with A.2 to find the surface potential,

Ψ_0 , and subsequently the interfacial ion concentrations and IDA species concentrations.

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Chapter 3

**Protein targeting to metal-chelating lipid monolayers
via naturally occurring and engineered histidines.**

Preface

Parts of the following chapter are adapted from a previous publication. The experiments of cytochrome b_5 binding to PSIDA-Cu lipid were discussed in K. Ng, D.W. Pack, D.Y. Sasaki and F.H. Arnold, "Engineering Protein-Lipid Interactions: Targeting of Histidine-Tagged Proteins to Metal-Chelating Lipid Monolayers," *Langmuir* **11**, 4048-4055, 1995 (©1995 American Chemical Society).

The cytochrome b_5 -PSIDA-Cu monolayer work (excluding the fluorescence microscopy) was performed in collaboration with Dr. Kingman Ng. I designed the experimental apparatus and Dr. Ng and I, together, designed the experiments. Dr. Ng performed the experiments while I performed the analysis of the data including development of the equilibrium binding model.

Introduction

The biological membrane, consisting of a wide variety of proteins dispersed within a lipid matrix, is a functional structure responsible for performing a wide variety of complex tasks within the living cell. For example, membrane targeting of soluble ligands accompanied by reorganization and assembly of the membrane components is a fundamental feature of cellular signal transduction [1] and intercellular communication [2]. Further, targeting followed by assembly into superstructures underlies a variety of other biological processes ranging from assembly of the prothrombinase complex and blood coagulation [3], to biomineralization [4-6] and the formation of two-dimensional protein crystals on bacterial cell surfaces [7, 8]. Protein or lipid components provide the interactions required for targeting and orientation of ligands on the membrane surface while the intrinsic membrane fluidity allows reorganization and assembly into functional supramolecular structures.

The ability to mimic these membrane processes in artificial lipid-membrane systems leads to a wide variety of applications in fields ranging from biotechnology to materials science. For instance, protein assemblies in synthetic membranes are of considerable use for drug delivery systems [9-11], biosensors [12, 13], biomolecular devices [14, 15], synthesis of novel organic and inorganic materials [16, 17] and model systems for more complex biological structures [18, 19]. Simply controlling protein orientation on a surface has been shown to be useful for AFM imaging [20], stabilization of labile biomolecules [21], obtaining novel electronic and optical properties [4], and influencing subsequent recognition processes [22]. Thus, the goal of this research has been to develop simple, robust materials capable of reproducing some of the important aspects of biological membrane targeting, reorganization and assembly.

A plethora of strategies for protein targeting to and immobilization on surfaces has been described in the literature. The most simple methods employ non-specific interactions

such as electrostatics [23, 24]. In these cases, little control over the resulting surface is possible, however. A variety of more sophisticated schemes allowing more control over adsorption and orientation have been developed as well. For instance, covalent immobilization of proteins has been achieved using N-hydroxysuccinimidyl ester-functionalized self-assembled monolayers [25] and photoactive surfaces functionalized with benzophenone [26] or phenyl azido groups [27, 28]. Each of these schemes targets surface amines (i.e. lysines) and, therefore, specificity of the reaction site on the protein surface and protein orientation are limited. Sligar and co-workers have developed two schemes, using iodopropyl- [29] and maleimido-derivatized [30] surfaces, which target unique surface thiols (i.e. cysteine) thereby allowing homogeneous orientation of the bound protein.

The ability to mimic biological membrane reorganization and assembly requires a dynamic surface, usually a lipid mono- or bilayer. Organization of proteins on artificial lipid membranes is usually mediated by interactions with specific lipid-bound receptors or affinity ligands. Probably the most noted example of this approach is the targeting and two-dimensional crystallization of streptavidin on biotin-functionalized lipid layers (c.f. Chapter 4) [19, 31]. Although the biotin-streptavidin system exhibits very strong and specific binding, the range of proteins that can be bound to the membrane surface and the types of molecular architectures produced are limited. Other affinity ligands which have been used to target proteins to model membranes include haptens for antibodies [32-35], antibodies for specific proteins [9, 11], inhibitors/effectors for enzymes [36, 37] and the heme prosthetic group for apomyoglobin [38]. This approach has two inherent disadvantages, however. First, it is necessary to design and synthesize a unique affinity lipid for each new protein of interest. Perhaps more importantly, no affinity ligands are available or even known for many proteins with interesting biological, optical, electronic or catalytic properties. Thus, a versatile, generally-applicable, affinity system for targeting and orienting proteins at membranes would be extremely useful.

The specific interaction between the amino acid histidine and divalent transition metal ions, as described in Chapter 1, provides one such affinity system. This interaction has been utilized for a number of years for protein purification *via* immobilized metal-ion affinity chromatography (IMAC) [39, 40]. Coordination to divalent transition metal ions, e.g., Cu^{2+} or Ni^{2+} , by the lone pair of electrons on either of the imidazole nitrogens of the histidyl side chain provides an interaction that is relatively strong ($K_a \sim 10^{3.5} \text{ M}^{-1}$ for imidazole coordination to Cu^{2+} -iminodiacetate (IDA-Cu) [41, 42]), at least in comparison to a hydrogen bond or charge-charge interaction. Much higher affinities, comparable to those of specific biological recognition systems, can be obtained when surface-bound metal ions simultaneously coordinate multiple functional groups on a macromolecular ligand (e.g. several histidines on the surface of a protein) [40, 43]. Studies with engineered proteins containing as few as two surface-accessible histidines have shown that multipoint attachment results in apparent protein binding constants greater than 10^6 M^{-1} [44]. Furthermore, proteins can be engineered to display high-affinity metal-binding sites [45, 46] or metal-binding peptides (e.g. hexa-histidine tags) at their N- or C-terminus [43, 47], extending this approach to virtually any protein of interest.

To utilize the copper-histidine interaction for targeting proteins to lipid membranes, the metal ions must be immobilized on the membrane surface yet accessible for protein binding. Several chelating lipids that can form ternary lipid-metal-protein complexes have been designed and studied in our laboratory (c.f. Chapter 2). The two lipids used in the present study, 1-octadecyl-2-(9-(1-pyrene)nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid) (PSIDA) [48] and 1,2-distearyl-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid) (DSIDA) [49], are shown in Figure 3.1.

In the present work, targeting of two proteins, myoglobin and cytochrome b_5 , to metal-chelating lipid monolayers is described. The surface pressure-molecular area (π -A) behavior of the lipid monolayers, in the presence and absence of protein, was observed in order to qualitatively examine specific protein binding. In addition, fluorescence

microscopy provides direct physical evidence of protein binding by visualizing the distribution of copper-chelating lipids and bound protein, simultaneously. Both naturally occurring histidines (myoglobin) and the hexa-histidine affinity tag (cytochrome b_5) were found to be effective at targeting protein to the membrane surface. In fact, the hexa-histidine tag bound protein to the metal-chelating monolayer with high affinity; an apparent association constant of $2 \times 10^7 \text{ M}^{-1}$ was estimated. The IDA-Cu-histidine interaction provides an effective and versatile system for targeting proteins to lipid assemblies.

Results

Metal-chelating DSIDA lipid monolayers were prepared by spreading the metal-free lipid on buffered subphase containing the appropriate metal salt, as described in Chapter 2. In addition to the copper-chelating monolayers, calcium-chelating monolayers were prepared as non-binding, control membranes (*vide infra*). Just as with CuCl_2 , a contraction of the monolayer π -A isotherm can be effected upon addition of CaCl_2 to the subphase (Chapter 2), although much higher concentrations of CaCl_2 are required. At $100 \text{ } \mu\text{M}$ CaCl_2 (Figure 3.2) the π -A isotherm is similar to the curve of DSIDA on $0.35 \text{ } \mu\text{M}$ CuCl_2 (Figure 2.2). The π -A curve of DSIDA on the same buffered subphase containing 1 mM CaCl_2 is *identical* to the DSIDA-Cu π -A curve suggesting the IDA headgroups are saturated with Ca^{2+} at this concentration. In addition, the π -A isotherm of DSIDA on a buffered subphase containing *both* $10 \text{ } \mu\text{M}$ CuCl_2 and 1 mM CaCl_2 is identical to the π -A isotherms in the presence of either metal alone. Due to the relative affinities of IDA for Cu^{2+} and Ca^{2+} , we can expect this monolayer to consist predominantly of DSIDA-Cu (*vide infra*).

Targeting of horse heart myoglobin to IDA-Cu lipid monolayers.

As discussed in the previous chapter, qualitative features of ligand binding to metal-chelating lipids can be discerned from changes in the π -A isotherms of monolayers of the lipids spread at the air-buffer interface. Monolayers of DSIDA spread on a buffered subphase containing the appropriate metal were compressed to 5 mN/m, and a concentrated solution of horse heart myoglobin was injected into the subphase behind the barriers to give a final protein concentration of $10 \pm 1 \mu\text{M}$. The monolayers began to expand immediately after injection. Surface pressure-area isotherms for DSIDA-Cu and DSIDA-Ca (Figure 3.3, curves a and b) were measured after the monolayers were allowed to expand to 80 $\text{\AA}^2/\text{molecule}$ at constant pressure. (The choice of 80 $\text{\AA}^2/\text{molecule}$ for the beginning of the isotherm is arbitrary. When allowed, the monolayers continued to expand until the trough area reached its maximum. At this point, the surface pressure began to rise due to further insertion of protein.) In each of these experiments, the isotherm measurements were initiated at the same surface pressure (5 mN/m), with the same area available to each lipid molecule (80 $\text{\AA}^2$) and equal amounts of lipid in each monolayer. Thus, the increase in area relative to the identical monolayer in the absence of protein and at an equivalent surface pressure (e.g. curve c) is attributed to protein insertion, and the two metal-containing monolayers presumably contain the same amount of myoglobin when compression begins.

The π -A isotherm of DSIDA-Cu in the presence of myoglobin (Figure 3.3, curve a) shows a steep increase in surface pressure at high molecular areas, followed by a region of high compressibility at 25-30 mN/m and a highly condensed region with a limiting area of 51 $\text{\AA}^2/\text{molecule}$. In contrast, the DSIDA-Ca isotherm (Figure 3.3, curve b) exhibits greater compressibility at high areas and a less pronounced transition region. At low molecular areas, this monolayer is much more compressible than the Cu^{2+} -containing monolayer and exhibits a larger limiting area equal to 55 $\text{\AA}^2/\text{molecule}$.

The effect of binding site density on protein targeting was studied using mixed monolayers of DSIDA with 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC). Isotherms of mixed monolayers of DSIDA-Cu/DSPC and DSIDA-Ca/DSPC (1:19) in the presence of myoglobin, shown in Figure 3.4, are similar to those of the corresponding monolayers of pure, metalated DSIDA. As for the DSIDA-Cu monolayer, the DSIDA-Cu/DSPC (1:19) monolayer on the protein-containing subphase (curve a) is significantly expanded, even at high pressure, relative to the same monolayer in the absence of protein (curve c). The presence of myoglobin increases the molecular area at 50 mN/m from 39 to 45 Å²/molecule. The DSIDA-Ca/DSPC (1:19) monolayer (curve b) is also more expanded at high pressure, but to a lesser degree (molecular area = 42 Å²/molecule at 50 mN/m).

The metals also have different effects on the kinetics of protein association with the monolayers. Surface pressure-molecular area isotherms of DSIDA-Cu and DSIDA-Ca monolayers, begun 5 minutes after protein injection, are shown in Figure 3.5. The isotherm of pure, metalated DSIDA in the absence of protein is shown for comparison. After 5 minutes at 5 mN/m, the DSIDA-Cu monolayer expanded by 35 Å²/molecule, while the DSIDA-Ca monolayer expanded by only 10 Å²/molecule. [Full expansion of the DSIDA-Ca monolayer to 80 Å²/molecule required approximately 15 minutes (Figure 3.3).] Expansion of the mixed DSIDA/DSPC (1:19) monolayers to the same area was somewhat slower. Nine minutes after addition of myoglobin, the DSIDA-Cu/DSPC (1:19) monolayer expanded by 32 Å²/molecule, while the molecular area of the DSIDA-Ca/DSPC (1:19) monolayer increased by only 14 Å²/molecule, relative to the metalated DSIDA/DSPC (1:19) monolayer in the absence of protein (Figure 3.6). [Full expansion of the DSIDA-Ca/DSPC (1:19) monolayer to 80 Å²/molecule required 23 minutes (Figure 3.4).]

Targeting of hexa-histidine tagged cytochrome cyt b₅ to IDA-Cu lipid monolayers.

Proteins with only few or no surface histidines can be conferred with the ability to strongly bind immobilized metal ions by, among other routes, the addition of a hexa-histidine fusion peptide genetically engineered at the N- or C- terminus [43]. Here we show that the soluble domain of rat liver cytochrome b₅ engineered to contain a hexa-histidine tag at the C-terminus (6-His cyt b₅) can be targeted with high affinity to metal-chelating lipid monolayers. The wild type protein (wt cyt b₅) serves as a non-binding control.

As above, qualitative features of protein binding to lipid monolayers were observed from changes in the monolayer π -A behavior. These studies were carried out in order to demonstrate specific targeting *via* metal-ion coordination and distinguish it from nonspecific protein insertion into the monolayer. Nonspecific protein insertion, observed for a variety of protein-lipid systems at low surface pressures [50, 51], can be minimized by increasing the surface pressure. To identify conditions that favor specific protein targeting, a pure, metal-free PSIDA monolayer was compressed and held at constant pressure while the monolayer relaxed (15-30 min.). Upon addition of 6-His cyt b₅ to the subphase (final concentration ~ 200 nM), measurable increases in monolayer surface area due to nonspecific insertion were observed only at surface pressures less than 10 mN/m. All subsequent cyt b₅ binding experiments were carried out at 25 mN/m, well above this threshold pressure.

Specific protein binding involves formation of a ternary complex between the IDA lipid, the metal ion and the poly(histidine) fusion peptide. To establish that 6-His cyt b₅ binding occurs *via* this mechanism and not through other nonspecific interactions with the membrane, protein-induced monolayer area changes were measured at constant pressure for three situations: (1) wild-type protein (no 6-His) with IDA-Cu lipid; (2) 6-His protein with IDA lipid (no Cu²⁺) and (3) 6-His protein with the IDA-Cu lipid. No monolayer

expansion was observed when 6-His cyt b_5 was injected below a PSIDA monolayer that had not been metalated, while a large increase in area is seen for the pre-metalated monolayer (Figure 3.7). Subsequent addition of an excess of a strong metal-chelating agent, ethylenediaminetetraacetate (EDTA), to the expanded monolayer caused the area to decrease (data not shown). The area did not decrease to the initial area. However, this is to be expected as the molecular area of unmetalated PSIDA is larger than that of PSIDA-Cu. Although 6-His cyt b_5 does not insert into unmetalated PSIDA at 25 mN/m (see above), it is still possible that the monolayer expansion in the presence of Cu^{2+} is due to nonspecific interactions with the monolayer, as Cu^{2+} changes the nature of the membrane surface (see Chapter 2). This possibility can be excluded, however, by comparing binding of the histidine-tagged protein and wt cyt b_5 (no 6-His) to the PSIDA-Cu monolayer. Without the poly(histidine) fusion peptide, no change in monolayer area was observed.

The effect of the monolayer structure on the observed area increase was examined by comparison of the PSIDA lipid to DSIDA, which forms less fluid, solid-like monolayers. Similar results were obtained upon addition of 6-His cyt b_5 to the DSIDA-Cu monolayer, although the extent of monolayer expansion is much less than for 6-His cyt b_5 binding to PSIDA-Cu (Figure 3.8). As with PSIDA, no area change was observed for wt cyt b_5 binding to DSIDA-Cu, nor did the His-tagged protein affect the area of the unmetalated monolayer (data not shown).

Figure 3.9 shows the PSIDA-Cu monolayer expansion measured for different concentrations of 6-His cyt b_5 in the subphase ranging from 50 nM to 1 μM . In order to maintain reproducibility and facilitate comparison among experiments, care was taken to ensure that the same amount of PSIDA-Cu lipid was spread in each experiment. The error in the initial monolayer area at $\pi = 25$ mN/m is about 2%. As the protein concentration is reduced from 1 μM to 50 nM, the final extents of monolayer expansion remain similar. The time required to reach maximum adsorption, however, increases significantly with decreasing protein concentration. That complete monolayer expansion is observed with as

low as 50 nM 6-His cyt b_5 in the subphase suggests an apparent dissociation constant, K_d , for protein binding of less than 50 nM ($K_a > 2 \times 10^7 \text{ M}^{-1}$).

The influence of interfacial metal-ion density on protein targeting was studied using mixed monolayers of PSIDA and a matrix lipid that does not chelate metal ions, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC). Considerations in choosing a matrix lipid were that it have little or no non-specific interactions with the protein, maintain the fluidity of the monolayer and be miscible with PSIDA so that the metal ions would be dispersed uniformly in the monolayer, at least initially. The zwitterionic phosphocholine headgroup of SOPC is expected to show little or no interaction with cyt b_5 in high salt buffer, while the oleoyl tail ensures monolayer fluidity at all surface pressures (at room temperature) [52]. Finally, PSIDA, both with and without chelated metal ion, appears to be readily miscible with SOPC at all compositions and a surface pressure of 25 mN/m as shown in the previous chapter.

Surface area measurements show no monolayer expansion when 6-His cyt b_5 is injected below pure SOPC, either with or without Cu^{2+} in the subphase (data not shown). In addition, no expansion of a monolayer containing 10 mol% PSIDA-Cu was observed in the presence of 200 nM 6-His cyt b_5 . An expansion of less than 10% was observed upon injection of the same amount of protein under a monolayer containing 30 mol% PSIDA-Cu. Above 30 mol% PSIDA-Cu, the amount of monolayer expansion increased with the amount of metal-chelating lipid in the film (Figure 3.10). The absence of expansion for the 10 mol% PSIDA-Cu monolayer is notable, as there is still a significant density of metal ions at the interface (~ 1 per $500 \text{ \AA}^2$) at this PSIDA concentration.

Fluorescent labeling of His-tagged proteins.

Fluorescence microscopy of monolayers and monolayer-bound ligands such as proteins provides a convenient means of visualizing the structure of the lipid layer and the distribution of protein in the system. Techniques for derivatizing proteins with fluorescent

labels have been known for some time and are now quite standardized [53, 54]. The treatment is generally very mild. Nevertheless, one should always ensure that the labeling procedure is benign with respect to the protein properties of interest.

I have found that labeling of 6-His cyt b_5 with fluorescein succinimidyl ester (FSE) according to the standard protocol (see Experimental) greatly interferes with binding of the protein to IDA-Cu-derivatized surfaces. Shown in Figure 3.11 are the retentions of native and labeled protein (Fl 6-His cyt b_5) in high performance liquid chromatography (HPLC) IMAC. The native 6-His cyt b_5 was retained quite strongly, eluting at 120 mM imidazole in a gradient from 20 to 250 mM imidazole over 54 minutes. In contrast, the Fl 6-His cyt b_5 eluted from the column as multiple peaks. Less than 20% of the protein eluted as the native 6-His cyt b_5 , approximately 50% eluted as multiple, unresolved peaks centered at 80 mM imidazole and the remainder, retained only very weakly or not at all, eluted in the void volume or slightly thereafter. It should be noted that protein in the eluent was detected by the heme absorbance at 413 nm. The fluorescein-derivatized protein could, in addition, be detected by absorbance of the fluorophore at 495 nm. The chromatograms appear identical at the two wavelengths indicating all fractions are labeled and contain approximately the same number of fluorescein groups per protein (data not shown).

Although the succinimidyl ester is only weakly reactive toward histidine, the most obvious explanation for the heterogeneous binding of the labeled protein is attachment of the label on or near the histidine tail which then interferes with binding to the IDA-Cu-derivatized support. However, this can not be the case as binding of 6-His cyt b_5 , which was subjected to the standard labeling procedure omitting the addition of FSE, is very similar to that of the labeled protein (data not shown). Therefore, we must conclude that the protein is damaged by some aspect of the labeling procedure and not by the reaction with FSE itself. In order to isolate which step of the labeling procedure is responsible for the negative effect on protein binding, three individual aliquots of the protein were incubated for one hour under one of the following conditions mimicking individuals steps

of the reaction: (1) pure labeling buffer at room temperature, (2) labeling buffer plus 6% DMF and (3) labeling buffer plus 0.15 M hydroxylamine. Each sample was then analyzed by HPLC IMAC for its ability to bind the metal-chelating surface. The samples incubated in pure labeling buffer and labeling buffer plus DMF eluted from the column in a single peak similarly to the native 6-His cyt b₅, indicating no damage to the protein. In contrast, the sample incubated in the presence of hydroxylamine eluted as multiple peaks in a pattern identical to that observed for the Fl 6-His cyt b₅ (Figure 3.12). Thus, it appears that the hydroxylamine is responsible for the heterogeneous reduction in binding to the IMAC support. The mechanism of hydroxylamine-induced damage is not known. Two possibilities are nucleophilic attack on the imidazole rings of the histidine side chains or cleavage of all or part of the hexa-histidine tail from the protein.

Hydroxylamine is added to the reaction mixture to neutralize unreacted FSE, effectively quenching the reaction, and to remove weakly bound fluorophore from the protein prior to separation of unreacted FSE and protein by size exclusion chromatography (SEC). As such, this step is not strictly necessary for efficient labeling and was subsequently removed from the procedure. Protein derivatized without the hydroxylamine quenching step, in fact, shows only small amounts of the weaker binding fractions with greater than 80% of the protein recovered in the strong binding peak eluted at 120 mM imidazole (Figure 3.13). This labeling procedure was used for all subsequent derivatizations of protein with FSE or rhodamine succinimidyl ester (RSE).

Fluorescence microscopy of monolayer-bound protein.

Copper ions are known to quench a wide variety of fluorophores. For example, the quenching of Fl 6-His cyt b₅ by Cu²⁺ is demonstrated in Figure 3.14. The plot of F₀/F versus copper concentration (Stern-Volmer plot) curves downward (second derivative < 0), most notably at high [Cu²⁺]. In contrast, Ni²⁺ ions show very little quenching of the labeled protein. For this reason, IDA-Ni monolayers have been used in imaging lipid-

bound protein by fluorescence microscopy. The IDA-Ni complex binds imidazole with somewhat lower affinity than IDA-Cu ($\log K = 2.78$ for IDA-Ni while $\log K = 3.38$ for IDA-Cu [41, 55]). Nevertheless, both Ni^{2+} and Cu^{2+} serve to bind His-tagged proteins very tightly to IDA-derivatized supports in IMAC [40], and the IDA-Ni headgroups should provide a good surface for binding His-tagged proteins.

Measurements of the surface pressure versus area, described above, provide indirect evidence of protein targeting to the metal-chelating lipid monolayers. We have also obtained direct physical evidence of specific protein binding using fluorescence microscopy. The distribution of 6-His cyt b_5 on the monolayer was visualized by labeling the protein with the fluorescent marker RSE (Rh 6-His cyt b_5). A fluorescence micrograph of a supported monolayer of a 1:1 mixture of PSIDA-Ni and 1,2-dibehenoyl-*sn*-3-glycero-phosphocholine (DBPC) viewed through pyrene excitation and emission filters is shown in Figure 3.15a. Due to the difference in length of the tails, these lipids are expected to phase separate. In fact, the fluorescence micrograph reveals dark domains corresponding to DBPC-rich regions on a bright background of predominantly PSIDA-Ni lipid. The pyrene label is excited at wavelengths in the near UV (ca. 355 nm), while rhodamine is excited at much higher wavelengths (535 nm). This difference allows the monolayer and the protein to be imaged independently. Figures 3.15a (pyrene) and 3.15b (rhodamine) show the same pattern of contrast; little rhodamine fluorescence is observed from the domains which are dark in the pyrene image while a strong rhodamine signal emanates from the bright, pyrene-containing background regions. From these data, we can conclude that the PSIDA-Ni lipid and the Rh 6-His cyt b_5 are localized to the same regions of the monolayer. In the absence of metal ion, very little rhodamine fluorescence was observed at the monolayer-solution interface (data not shown).

As described above, binding of 6-His cyt b_5 to PSIDA/SOPC mixed monolayers was observed by surface pressure-area measurements only at PSIDA contents greater than or equal to 30 mol%. Surprisingly, at 10 mol% PSIDA no change in monolayer area was

observed. Metal ion-mediated protein targeting to the PSIDA/SOPC interface at each of these IDA-Cu lipid concentrations was confirmed by fluorescence microscopy of the fluorescein-labeled 6-His cyt b_5 . Figure 3.16 compares the FI 6-His cyt b_5 signals from three mixed PSIDA/SOPC monolayers (10, 30 and 100 mole % PSIDA), in the presence and absence of metal ions. Care was taken to ensure identical camera settings from experiment to experiment so that fluorescence intensity can be correlated with fluorophore, and therefore protein, density. In the presence of Ni^{2+} , a uniformly bright field is observed. The fluorescein fluorescence intensities of the 100% and 30% PSIDA monolayers are similar, while the 10% PSIDA monolayer is somewhat less intense. Nevertheless, protein is targeted to the 10 mol% PSIDA-Ni monolayer even though no area change was observed. In the absence of Ni^{2+} , very little fluorescein emission was observed for any of the three monolayers. Nor was any fluorescence signal detected from a pure SOPC monolayer in the presence of 10 μM NiCl_2 and FI 6-His cyt b_5 , indicating that the phosphocholine headgroup alone is unable to target the protein to the monolayer (data not shown).

Discussion

In the course of investigating protein targeting to metal-chelating lipid monolayers, we have utilized IDA lipids loaded with Ca^{2+} in place of Cu^{2+} as a non-binding control surface. Calcium-loaded lipids provide an excellent control for two reasons. First, the IDA headgroups chelate Ca^{2+} similarly to Cu^{2+} , albeit with lower affinity ($\log K_{\text{Ca-IDA}} = 2.6$ M^{-1}) [55], resulting in a charge neutral interface with physical properties very similar to those of the IDA-Cu monolayers. Second, imidazole is not a good ligand for Ca^{2+} , exhibiting a weak association constant in solution of 1.2 M^{-1} [56].

Calcium-containing monolayers were prepared by spreading the DSIDA lipid on buffered subphase containing CaCl_2 . The changes in shapes of the π -A isotherms upon

titration of the subphase with CaCl_2 are very similar to those observed upon titration with CuCl_2 , suggesting the binding of the two metals is occurring in a similar fashion. In addition, the π -A isotherm of DSIDA in the presence of 1 mM CaCl_2 is identical to that in the presence of 10 μM CuCl_2 , suggesting that the lipid is saturated with Ca^{2+} at this subphase concentration. That saturation of the monolayer requires a much higher concentration of CaCl_2 than CuCl_2 is to be expected given the relative affinities of IDA for the two metal ions in solution ($\log K_{\text{Cu-IDA}} = 10.6$ versus $\log K_{\text{Ca-IDA}} = 2.6 \text{ M}^{-1}$) [55]. Note that due to the high local concentration of IDA at the interface and the presence of the charge double layer, the stability constants for metal complexation at the interface differ from those in the bulk (Chapter 2). However, the *relative* affinities for different ions should not change greatly. The isotherm of DSIDA on buffered subphase containing both 10 μM CuCl_2 and 1 mM CaCl_2 is identical to that on a subphase containing either 10 μM CuCl_2 or 1 mM CaCl_2 , alone. Although the π -A curve does not indicate which metal is bound to the lipid under these conditions, we can reasonably expect the monolayer to consist almost exclusively of DSIDA-Cu, given the relative metal concentrations and their affinities for IDA ($K_{\text{Cu-IDA}}[\text{Cu}^{2+}] = 10^{5.6}$ versus $K_{\text{Ca-IDA}}[\text{Ca}^{2+}] < 1$).

The Gouy-Chapman-Stern theory of the diffuse double layer at charged interfaces [57, 58] provides a means of calculating surface charge densities and species distributions of the lipid headgroups as described in Chapter 2 and, therefore, provides a means of predicting the metalation of IDA lipids with Cu^{2+} and Ca^{2+} . The analysis provided in the previous chapter can be extended to include CaCl_2 in the subphase resulting in the following two independent equations for the surface charge density, σ :

$$\sigma = - \left[2\epsilon_r \epsilon_0 kT \sum_i [c_i]_{\infty} \left\{ \exp \left(- \frac{z_i e \Psi_0}{kT} \right) - 1 \right\} \right]^{1/2} \quad (1)$$

and

$$\sigma = \frac{\left(\frac{e}{A}\right)\left(2 + \left(K_{a1}[H^+]_{\infty} + K_{Na}[Na^+]_{\infty}\right)\Phi - K_{a1}K_{a2}K_{a3}[H^+]_{\infty}^3\Phi^3\right)}{1 + \left(K_{a1}[H^+]_{\infty} + K_{Na}[Na^+]_{\infty}\right)\Phi + \left(K_{a1}K_{a2}[H^+]_{\infty}^2 + K_{Cu}[Cu^{2+}]_{\infty} + K_{Ca}[Ca^{2+}]_{\infty}\right)\Phi^2 + K_{a1}K_{a2}K_{a3}[H^+]_{\infty}^3\Phi^3} \quad (2)$$

where ϵ_r and ϵ_0 are the dielectric constants of water and vacuum, respectively; k is Boltzmann's constant; T is absolute temperature; e is the electronic charge; $[c_i]_{\infty}$ and z_i are the bulk concentration and valence of ionic species i , respectively; A is the molecular area of the lipids in the monolayer; K_{a3} , K_{a2} and K_{a1} are the protonation constants as shown in Figure 2.19; K_{Na} , K_{Ca} and K_{Cu} are the association constants of the IDA headgroups at the air-water interface with Na^+ , Ca^{2+} and Cu^{2+} , respectively; $[H^+]_{\infty}$, $[Na^+]_{\infty}$, $[Ca^{2+}]_{\infty}$ and $[Cu^{2+}]_{\infty}$ are the ion concentrations in bulk solution and $\Phi = \exp(-e\Psi_0/kT)$ in which Ψ_0 is the surface potential. Upon solving these two equations for σ and Ψ_0 , the interfacial ion concentrations, surface charge density and lipid species distributions can be determined as described in Chapter 2. In the absence of $CuCl_2$, the theory predicts the IDA headgroups are only 88% loaded with Ca^{2+} at 1 mM $CaCl_2$ in the subphase and using the association for Ca^{2+} and IDA in bulk solution, $\log K_{Ca-IDA} = 2.6 \text{ M}^{-1}$. However, it appears from the π -A isotherms that the IDA headgroups are saturated at this concentration. The reason for this discrepancy is unclear. In the presence of *both* 10 μM $CuCl_2$ and 1 mM $CaCl_2$ the theory predicts 94% to 99% of the headgroups are loaded with Cu^{2+} given $\log K_{Cu-IDA} = 6$ to 7 M^{-1} (as determined in Chapter 2). Thus, the theory predicts that our control surface consists primarily of IDA-Ca headgroups, and in the presence of both ions in the subphase the surface consists of IDA-Cu as desired.

Protein targeting to IDA-Cu lipid monolayers via naturally occurring histidines.

Horse heart myoglobin ($M_r = 17,600$), a water-soluble heme-containing protein, was chosen as a model protein for targeting to metal-chelating membrane surfaces *via* its

naturally occurring surface histidines (Figure 3.17). Heme-containing proteins are attractive candidates for production of functional protein-membrane materials due to the optical and electronic properties of the heme prosthetic group. Horse myoglobin contains eleven histidines, at least four of which can coordinate to IDA-Cu [59] (c.f. Table 3.1). Note that the crystal structure of horse heart myoglobin has been solved [60, 61], but the coordinates are not currently available. Therefore, we have used the coordinates for sperm whale myoglobin [62] for generating Figure 3.17 and for determining the histidine side chain accessibilities. The two sequences are highly homologous [63], sharing 88% sequence identity, and only minor errors may be expected from this substitution.

Myoglobin has been immobilized on the surface of lipid bilayers and cast lipid films by both non-specific electrostatic and specific binding mechanisms. Electrostatic association of the protein with lipid bilayers resulted in a fixed orientation, and the assembly was incorporated into a functional electron transport chain [23]. Myoglobin was specifically oriented on lipid bilayers by reconstitution of the apo-protein with a lipid-tagged proto-porphyrin derivative which anchored the protein to the membrane [38]. While these approaches work reasonably well for myoglobin, the copper-histidine interaction provides a much more general technique for protein targeting to lipid surfaces. We have not, to this point, examined protein orientation. However, oriented IDA-Cu-histidine binding is possible, especially for genetically engineered proteins on which multiple histidine residues on a single protein face can be introduced.

Surface pressure-molecular area isotherms have been used to investigate the targeting of myoglobin to metal-chelating DSIDA lipid monolayers. Because monolayers of DSIDA-Cu and DSIDA-Ca produce identical π -A behavior in the absence of protein, differences in the isotherms of the Cu^{2+} - and Ca^{2+} -containing monolayers upon addition of protein highlight the effects of specific protein-copper interactions. The constant-pressure expansion upon protein injection is most likely due to insertion of myoglobin into the lipid

films. Nonspecific protein insertion into lipid monolayers at low surface pressure has been observed for a variety of protein-lipid systems [50, 51, 64, 65]. Inclusion of Cu^{2+} in the lipid monolayers, however, significantly increases the rate of myoglobin insertion with respect to the Ca^{2+} -containing film. Because the identity of the metal has no effect on the films in the absence of protein, the rate enhancement in the Cu^{2+} -chelating systems may be attributed to an increase in protein concentration near the interface due to interactions with the bound copper ions.

The regions of high compressibility at large areas indicate that the protein is being "squeezed out" of the lipid monolayer [51, 66]. A large fraction of the protein appears to be ejected from the films at ~ 30 mN/m. Above this pressure, the monolayer of pure DSIDA-Ca remains more expanded with respect to the same monolayer in the absence of protein. Because the strong coordination interactions are not available in the film containing calcium, we believe this residual expansion reflects a small fraction of myoglobin that remains nonspecifically at the interface. Above 30 mN/m, the DSIDA-Cu film exhibits still higher areas at a given pressure, with a compressibility similar to that of the film without protein. The enhanced protein binding can be attributed to specific interactions with the lipid-bound copper. This further expansion could result from repulsion between protein molecules bound at the film surface. Alternatively, some of the protein molecules incorporated within the monolayer at low pressure could remain irreversibly bound within the Cu^{2+} -containing monolayer at high pressures (c.f. Figure 3.18).

A complimentary study of myoglobin binding to DSIDA-Cu-containing small unilamellar vesicles has been performed in our laboratory [49]. The protein was found to bind to vesicles containing 2 mol% DSIDA-Cu with a binding constant greater than $2.9 \times 10^6 \text{ M}^{-1}$. Myoglobin binding to DSIDA-Ca-containing vesicles was at least an order of magnitude weaker. Further, electron spin resonance spectroscopy of the copper loaded on the vesicles, in the presence of native myoglobin and myoglobin whose surface histidines

had been derivatized with DEPC (diethylpyrocarbonate), demonstrated the primary role of the histidines in protein targeting *via* coordination to membrane-bound metal ions.

Quenching of fluorescently labeled, hexa-histidine-tagged proteins.

Cu^{2+} in solution was found to quench fluorescein-labeled 6-His cyt b_5 . Both static and dynamic quenching mechanisms individually produce linear Stern-Volmer plots. When both mechanisms are present simultaneously, the Stern-Volmer plot curves upwards away from linearity. However, in the present case the curve is concave downward toward the x-axis. This behavior is generally indicative of two populations of fluorophores, one of which is not accessible for quenching. The Stern-Volmer equation can be modified to take account of a non-accessible fraction to give [67]

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K [Q]} + \frac{1}{f_a} \quad (3)$$

where F_0 is the fluorescence intensity in the absence of quencher, $\Delta F = F_0 - F$, $[Q]$ is the concentration of quencher, K is the Stern-Volmer quenching constant and f_a is the fraction of fluorophores accessible to quencher. The modified Stern-Volmer plot for quenching of Fl 6-His cyt b_5 by Cu^{2+} is shown in Figure 3.19. From the linear least squares fit to the data, we can determine $K = 130 \mu\text{M}^{-1}$ and $f_a = 0.63$. That some fraction of the fluorescent label would be inaccessible to quenching is rather surprising as the labeling reaction targets primarily surface lysines, and therefore the fluorophores should be predominantly on the surface of the protein.

Quenching of the labeled protein by Cu^{2+} is significant, if only at copper concentrations in the high micromolar range. However, at the surface of an IDA-Cu lipid monolayer, the local chelated metal concentration is very high, on the order of several millimolar. Therefore, quenching of fluorescein-labeled protein bound to the lipids may be significant. For this reason we chose to utilize Ni^{2+} -chelating lipids for the fluorescence microscopy studies whenever possible. As will be seen in later chapters, fluorescence

images of labeled protein on IDA-Cu monolayers can be obtained. The degree of quenching of bound protein most likely depends on factors such as the location of the labeling site with respect to the IDA-Cu binding site and the degree of mobility in the bound protein. These factors are difficult to predict *a priori*, and use of IDA-Ni lipids is recommended where possible.

Protein targeting to IDA-Cu lipid monolayers via hexa-histidine affinity tags.

Metal-chelating lipids can provide a truly general method for targeting proteins to lipid surfaces *via* surface-accessible histidine side chains. Proteins which lack suitable, naturally occurring, histidine residues can be genetically engineered to contain a hexa-histidine affinity tag at either the N- or C-terminus [43]. In fact, histidine tags provide a strong interaction with metal-chelating lipids, in general stronger than that observed for naturally occurring histidines. The 6-His cyt b_5 protein examined here provides one such example. The soluble portion of cytochrome b_5 contains five histidines (Figure 3.20). Two of them, 39 and 63, are coordinated to the heme Fe^{3+} and are unavailable for binding. The other three are near the surface of the protein, and His 26 and His 80 appear to be solvent accessible (c.f. Table 3.2). However, control experiments with wt cyt b_5 (Figure 3.7) have shown that these residues do not contribute to IDA-Cu lipid monolayer binding, at least at the low protein concentrations (200 nM) employed here.

The ability of 6-His cyt b_5 to selectively bind the metal-chelating lipids has been qualitatively visualized by fluorescence microscopy (Figure 3.15). It can be clearly seen that the Rh 6-His cyt b_5 is localized to the regions containing the PSIDA-Ni lipids and is largely excluded from the DBPC domains. This result demonstrates our ability to govern the organization and assembly of ligands bound to the lipid surface. In this case, the intrinsic pattern of the phase-separated PSIDA-Ni/DBPC monolayer controls the resulting pattern of adsorbed protein. Similar results for patterning of histidine-containing peptides

bound to metal-chelating monolayers have been obtained by our group [68] and by Tampé and co-workers [69].

Interactions between proteins and the lipid monolayer spread at the air-water interface were discerned from changes in the surface pressure-area behavior of the monolayer. The surface activity of most proteins, however, complicates the molecular interpretation of surface pressure-area measurements. One difficulty lies in distinguishing specific binding (*via* metal ion coordination in the present case) from nonspecific insertion of the protein into the monolayer, commonly observed at low surface pressures [50, 51]. Nonspecific insertion introduces protein domains in the lipid monolayer, disrupting the lipid packing and resulting in a large increase in monolayer area at constant pressure. Because 6-His cytochrome b_5 inserts nonspecifically in DSIDA and PSIDA monolayers only below 10 mN/m, monolayer expansion measured at 25 mN/m can be attributed to a different protein binding mechanism. Our surface area measurements clearly indicate that the lipid packing in metal-chelating monolayers is modified by binding the His-tagged protein (Figures 3.7 and 3.8). The surface area measurements and the fluorescence microscopy data, together with the fact that binding is reversible with the addition of EDTA to the subphase, support the view that protein binding occurs *via* coordination of the fusion peptide to the IDA lipid-bound metal ion. Both Cu^{2+} and Ni^{2+} are effective for protein targeting with tridentate IDA lipids, as they are for metal-affinity chromatography of His-tagged proteins [40].

Varying the protein concentration in the subphase by a factor of 20 has little effect on the final extent of PSIDA-Cu monolayer expansion (Figure 3.9). The lowest protein concentration examined, 50 nM, corresponds to ~6 nmol total protein in the subphase, which is still several-fold greater than the amount required to form a monolayer beneath the IDA-Cu lipid monolayer. (Based on the dimensions of cytochrome b_5 from its crystal structure, 30 Å x 22 Å x 22 Å [70], and a lipid monolayer area at 25 mN/m of ~6000 nm², a protein monolayer would include ~2 nmol total protein). The fact that the system

exhibits essentially the same final protein-binding capacity at all concentrations studied suggests that the apparent K_d for IDA-Cu-6-His cyt b_5 binding at the interface is less than 50 nM.

The density of protein binding sites (metal ions) at the interface can be controlled by mixing the IDA lipid with a suitable matrix lipid, in the present case SOPC. PSIDA and SOPC were found to be readily miscible at $\pi = 25$ mN/m, both in the presence and absence of metal ion (see Chapter 2). Fluorescence microscopy of the bound protein also shows a homogeneous field (Figure 3.16), indicating protein and, presumably, IDA-Cu lipid are distributed throughout the film. Decreasing the PSIDA-Cu mole fraction in a mixed monolayer with SOPC decreases the total monolayer expansion caused by protein adsorption (Figure 3.10). However, the fluorescence microscope images indicate that the bound protein density is similar for monolayers containing 100 and 30 mol% PSIDA (Figure 3.16). That the 10 mol% PSIDA-Cu monolayer does not expand upon protein addition could indicate that the protein does not bind. This is unlikely, however, given the high affinity of the 6-His peptide for IDA-Cu and IDA-Ni complexes and the significant density of metal ions at the interface (~ 1 IDA-Cu per $500 \text{ \AA}^2$). The fluorescence microscopy experiments, in fact, show that fluorescein-labeled 6-His cyt b_5 is indeed targeted to SOPC monolayers containing 10 mol% PSIDA-Ni (Figure 3.16), albeit at a lower density than to monolayers containing 30 or 100 mol% PSIDA-Ni. Thus, we must conclude that while an increase in monolayer area reflects protein binding to the interface, protein binding does not necessarily change lipid packing and increase the surface area.

An explanation for these observations is offered below and outlined schematically in Figure 3.21. At very low fractions of PSIDA-Cu in mixed monolayers (Figure 3.21, frame A), the 6-His cyt b_5 occupies essentially all the binding sites at the interface. Bound protein molecules are expected to be well-separated, and the binding should be accurately described according to the Langmuir adsorption isotherm. The monolayer surface area is not significantly altered. As the fraction of PSIDA-Cu is increased, the density of bound

cyt b_5 increases with no change in monolayer area until the point at which the protein forms a complete monolayer beneath the lipid (frame B). The mole fraction of PSIDA-Cu in the monolayer, x_{IDA} , at this point equals A_L^0/A_p^0 , where A_L^0 is the mean molecular area of a lipid molecule and A_p^0 is the area occupied by a single protein molecule. The area per PSIDA-Cu molecule is $\sim 52 \text{ \AA}^2$ at 25 mN/m (obtained from the π -A isotherms, Chapter 2), while an estimate of A_p^0 obtained from the crystal structure (see above) is $\sim 500 \text{ \AA}^2$. Thus, for the current system $A_L^0/A_p^0 \sim 0.1$. Indeed, at $x_{IDA} = 10 \text{ mol\%}$ PSIDA-Cu, protein binding did not expand the monolayer while monolayers with higher fractions of PSIDA-Cu did expand. In monolayers containing higher PSIDA-Cu fractions, more metal ions than required for monolayer protein coverage are available at the interface. In order for protein to occupy these additional binding sites, area must be created at the interface (frame C). At PSIDA-Cu fractions greater than A_L^0/A_p^0 , the *density* of bound protein appears to remain essentially constant. The increase in final area with increasing fraction of PSIDA (Figure 3.11) is believed to reflect an increase in the *total* amount of bound protein. Under these conditions, the Langmuir isotherm no longer holds, as protein-protein and lipid-lipid interactions must be accounted for. The equilibrium protein capacity reflects a balance between the energetic cost of perturbing the lipid packing and the energetic benefit of additional IDA-Cu-histidine interactions.

The relative area change can be related to the fraction of IDA lipid in the monolayer (x_{IDA}) and the fraction of IDA lipids bound to protein (x_b), according to the proposed model. Initially, the monolayer area is given by $n_L A_L^0$, where n_L is the total number of lipids in the monolayer and A_L^0 is the average area per lipid (the mean molecular area). After addition of protein, and at IDA lipid fractions greater than A_L^0/A_p^0 , the new monolayer area is equal to the area of the bound protein layer, $n_p A_p^0$, where n_p is the number of bound proteins. Now, the relative area change is given by

$$\Delta A/A = (n_p A_p^0 - n_L A_L^0)/n_L A_L^0 = n_p A_p^0 / n_L A_L^0 - 1. \quad (4)$$

But, assuming one IDA lipid per protein, the number of bound proteins is equal to the number of IDA lipids bound to protein, $x_b x_{IDA} n_L$, and substituting into equation (4) yields

$$\Delta A/A = x_b x_{IDA} (A_p^0 / A_L^0) - 1. \quad (5)$$

Equation (5) allows prediction of the area change from the fraction of protein-bound IDA lipids or vice versa.

As mentioned above, at $x_{IDA} > A_L^0/A_p^0$, protein binding to the IDA monolayers is not expected to follow the Langmuir adsorption isotherm in which x_b is independent of the density of binding sites, x_{IDA} . In fact, we can see that for the present data x_b is strongly dependent on x_{IDA} (*vide infra*). Thus, a simple reaction-kinetic derivation of a Frumkin-type [71, 72] isotherm, which describes the 6-His cyt b_5 adsorption data reasonably well, will be described. The rate of adsorption of the protein to the IDA monolayer is assumed to be first order in the subphase protein concentration, C_p , and proportional to the concentration of free IDA lipids as follows,

$$\text{rate of adsorption} = k_{on} C_p n_L x_{IDA} (1 - x_b) \quad (6)$$

where k_{on} is the reaction rate constant for adsorption. The rate of desorption is first order in the concentration of bound protein,

$$\text{rate of desorption} = k_{off} n_p = k_{off} n_L x_{IDA} x_b \quad (7)$$

where k_{off} is the reaction rate constant for desorption. At equilibrium, the rates of adsorption and desorption are equal, and setting equations (6) and (7) equal establishes the equilibrium adsorption isotherm.

The rate constants, k_{on} and k_{off} , can be expressed with an Arrhenius dependence

$$k_{on} = k_{on}^0 \exp(-E_a / RT) \quad (8)$$

$$k_{\text{off}} = k_{\text{off}}^0 \exp(-E_d / RT) \quad (9)$$

where E_a and E_d are the activation energies for adsorption and desorption, respectively. If the rate of protein binding is independent of bound protein concentration and IDA lipid concentration ($E_a = E_a^0$, $E_d = E_d^0$), the Langmuir adsorption isotherm results upon inserting equations (8) and (9) into (6) and (7). In the traditional Frumkin isotherm, widely used to describe adsorption of organic species to metal electrode surfaces [73-75] and adsorption of soluble surfactants at gas-liquid interfaces [76, 77], the activation energies are expressed as linear functions of the amount of adsorbate bound. Similarly, in order to describe the present model, we can account for the perturbation of the lipid monolayer packing by letting the activation energies depend linearly on the increase in monolayer area as follows:

$$E_a = E_a^0 + a (A_L - A_L^0) \quad (10)$$

$$E_d = E_d^0 + b (A_L - A_L^0) \quad (11)$$

where A_L is the area per lipid after protein adsorption and a and b are constants.

Now the equilibrium condition can be written as

$$k_{\text{on}}^0 e^{-E_a^0 / RT} e^{-a(A_L - A_L^0) / RT} C_p (1 - x_b) = k_{\text{off}}^0 e^{-E_d^0 / RT} e^{-b(A_L - A_L^0) / RT} x_b. \quad (12)$$

Rearranging yields

$$K^0 C_p (1 - x_b) = x_b \exp[(a - b)(A_L - A_L^0) / RT] \quad (13)$$

where

$$K^0 = \frac{k_{\text{on}}^0 \exp(-E_a^0 / RT)}{k_{\text{off}}^0 \exp(-E_d^0 / RT)}. \quad (14)$$

We can further define an interaction parameter, κ , which characterizes the perturbation of the monolayer by the protein binding according to

$$\kappa = \frac{a-b}{RT} A_L^0. \quad (15)$$

Finally, substituting the interaction parameter into equation (13) yields, after rearrangement

$$x_b = \frac{K^0 C_p}{\exp\{[x_b x_{IDA} (A_L^0 / A_p^0) - 1]\kappa\} + K^0 C_p} = \frac{K^0 C_p}{\exp[(\Delta A / A)\kappa] + K^0 C_p}. \quad (16)$$

This adsorption isotherm relates the fraction of IDA lipids bound by protein to the protein concentration in the subphase and the mole fraction of IDA lipids in the monolayer. There are two adjustable parameters in this isotherm: K^0 , the intrinsic association constant (equivalent to the Langmuir adsorption binding constant which holds at $x_{IDA} < A_L^0 / A_p^0$), and κ , the interaction parameter. The interaction parameter characterizes the exponential reduction of the association constant with increasing perturbation of the lipid packing; i.e.

$$K = K^0 \exp[-\kappa(A_L - A_L^0)/A_L^0] = K^0 \exp[-\kappa(\Delta A/A)] \quad (17)$$

where K is the effective binding constant at a lipid area increase of $(A_L - A_L^0)$. In the absence of any perturbation of the lipid monolayer, $\kappa = 0$ and equation (16) reduces to the Langmuir adsorption isotherm.

The intrinsic association constant is expected to be a function of the particular protein-membrane interaction, while κ is dependent on the state of the lipid species (i.e. the identity of the lipid, surface pressure, temperature, etc.). Figure 3.22 demonstrates the dependence of the fraction of IDA lipid binding sites occupied, x_b , on each of these parameters for a range of IDA lipid mole fractions from 0.1 to 1.0. Not surprisingly, x_b increases with the reduced protein concentration, $K^0 C_p$, at a given value of κ . Further, x_b is seen to depend very strongly on the interaction parameter, κ , at values between 0 and 10.

At $\kappa = 0$, x_b is constant as expected, and increasing κ decreases x_b at all IDA lipid concentrations due to increased difficulty of creating area at the interface.

The data for adsorption of 6-His cyt b_5 to PSIDA-Cu monolayers at various IDA lipid fractions (Figure 3.10) have been plotted as x_b versus x_{IDA} in Figure 3.23. The best fit of equation (16) to the data is shown as a solid curve with $K^0 C_p = 14$ and $\kappa = 9.4$ ($R = 0.98$). The isotherm appears to fit the data reasonably well. An interaction parameter of 9.4 corresponds to a reduction in the association constant to $0.0091 \cdot K^0$ at $\Delta A/A = 0.5$ and $8.3 \times 10^{-5} \cdot K^0$ at $\Delta A/A = 1.0$. In these experiments, C_p is approximately 180 nM which gives a value for the intrinsic association constant of $7.8 \times 10^7 \text{ M}^{-1}$ ($K_d = 13 \text{ nM}$). This value compares favorably with the *apparent* K_d estimated above as 50 nM. It should be noted that the isotherm fit assumes a constant value of $K^0 C_p$ for all x_{IDA} . In our experiments, the *total* protein added was held constant. Since more protein is bound at higher fractions of x_{IDA} lipid, C_p actually decreases with increasing x_{IDA} . This provides one possible explanation for the discrepancy between the theory and experiment. However, the variation in C_p is only 3% from $x_{IDA} = 0.1$ to 1.0 resulting in only a small error in the isotherm. Other possible limitations in the model contributing to the discrepancy in the fit are (1) the simple linear dependence of the activation energy on the area increase and (2) the lack of accounting for protein-protein interactions in the activation energy. Nevertheless, the fit is reasonably good and, therefore, supports this simple physical model.

This proposed binding model is also consistent with the larger area increases observed for protein binding to metalated PSIDA than to DSIDA, at the same pressures and protein concentrations (Figure 3.8). From their π -A isotherms, it can be seen that these two lipids pack very differently at the air-buffer interface (Chapter 2). DSIDA-Cu forms very tightly packed, condensed monolayers, while PSIDA-Cu monolayers are more expanded and highly fluid. The fluid PSIDA-Cu monolayer readily reorganizes to allow

formation of the IDA-Cu-protein complex at the interface. More energy would be required to effect similar reorganization of the tightly-packed DSIDA-Cu monolayer. However, the surfaces of PSIDA-Cu and DSIDA-Cu monolayers are expected to be indistinguishable to protein binding; i.e. the 6-His tag “sees” very similar IDA-Cu derivatized surfaces in both cases. Therefore, the intrinsic binding constant, K^0 , should depend only on the specific interaction and will be the same for both lipids.

From the observed $\Delta A/A$ of 0.075, and using the $K_0 C_p = 14$ as determined for PSIDA-Cu, we can estimate $\kappa = 61$ according to equation (16). This large value of κ corresponds to an association constant of $5.7 \times 10^{-14} \cdot K^0$ at $\Delta A/A = 0.5$ (compared to $0.0091 \cdot K^0$ for PSIDA with $\kappa = 9.4$). Because of its smaller value of κ , the metalated PSIDA monolayer has a higher capacity for specific protein binding and, thus, is significantly more effective for targeting the His-tagged protein to the interface. As described above, κ characterizes the ability of the monolayer to be perturbed by increasing in area at constant surface pressure in response to lateral interactions between bound proteins. Thus, we might expect κ to be inversely correlated with the monolayer compressibility, $c = -(1/A)(dA/d\pi)$, which is a measure of the ability of the monolayer to change its packing in response to a change in surface pressure. In fact, the compressibilities of PSIDA-Cu and DSIDA-Cu at 25 mN/m are found to be 0.015 ($c^{-1} = 66$) and 0.0012 ($c^{-1} = 240$) m/mN, respectively.

Conclusion

Metal ion coordination by surface histidine residues is a promising strategy for targeting proteins to artificial membranes, monolayers at a gas-water interface and molecular assemblies deposited or self-assembled on solid substrates. Here we have shown that myoglobin, a small, globular protein containing four surface-accessible

histidines, is targeted specifically to IDA-Cu lipid monolayers. A wide variety of natural proteins can be immobilized on lipid monolayers in this fashion. In addition, with the use of genetically engineered histidines, especially poly(histidine) affinity tags, nearly any desired protein can be immobilized and possibly oriented on lipid surfaces. One such protein, 6-His cyt b_5 , has been shown to be targeted to PSIDA-Cu containing lipid membranes with relatively high affinity (apparent $K_a = 2 \times 10^7 \text{ M}^{-1}$) and specificity for the IDA-Cu headgroups. Thus, the metal-chelating lipids provide a versatile and generally-applicable approach for targeting proteins with very high affinity and high specificity to lipid assemblies.

Targeting of proteins to interfaces is important for a large variety of applications as discussed above. Another promising application for this targeting system is in the two-dimensional (2D) crystallization of proteins [78]. Previous studies indicate that the following conditions favor two-dimensional crystallization [33, 79, 80]: (1) fixing the molecules in a plane with a specific orientation, (2) high concentrations of the molecule at the interface and (3) allowing the molecules sufficient mobility within the plane to sample various intermolecular contacts. Langmuir monolayers can provide these conditions: an affinity ligand serves to concentrate the protein molecules at the interface and fix them in the monolayer plane with a particular orientation (conditions 1 and 2), while the fluidity of the monolayer allows the bound proteins to sample different intermolecular contacts (condition 3). The 2D crystallization of streptavidin on IDA-Cu lipid monolayers is described in Chapter 4. In addition, the same poly(histidine) fusion peptide used to purify a recombinant protein by metal-affinity chromatography could also serve as a general affinity tag for two-dimensional crystallization on monolayers of suitable metal-chelating lipids.

Experimental

Materials

The metal chelating lipids DSIDA [49] and PSIDA [48] were synthesized as described previously (see Chapter 2). The phosphocholines 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and were used as received without further purification. Chloroform, methanol, ethanol (all A.C.S. HPLC grade) and CuCl_2 (99.999%) were obtained from Aldrich (Milwaukee, WI). 3-(N-morpholino)propanesulfonic acid (MOPS) and horse heart myoglobin (> 95% pure) were obtained from Sigma (St. Louis, MO).

Monolayer studies of myoglobin-DSIDA-Cu interactions.

Pressure-area (π -A) isotherms were obtained on a computer-controlled Langmuir instrument (KSV Instruments, model M1200) with a surface area of 250 x 75 mm². The surface pressure was measured using a platinum Wilhelmy plate. For all experiments the subphase was buffered with 20 mM MOPS, 100 mM NaCl at pH 7.5, and the temperature was maintained at 24 ± 0.5 °C. Lipids were spread from CHCl_3 (~0.75 $\mu\text{mol/mL}$) as the free acid. Monolayers of lipid-metal complexes were prepared on subphase containing the appropriate divalent metal ion as the chloride salt. Approximately 40 μL of the lipid solution were spread with a Hamilton syringe and, after allowing 15 minutes for solvent evaporation, the monolayer was compressed at a constant rate of 3 $\text{\AA}^2/\text{molecule}\cdot\text{min}$ to a pressure of 5 mN/m. After a period of equilibration of ~15 minutes, 1 mL of 1 mM myoglobin solution was injected into the subphase at several points behind each barrier. The protein solution was distributed evenly throughout the subphase by circulation of

buffer from one end of the trough to the other by means of a peristaltic pump. With the instrument operating in constant-pressure mode, the monolayer was allowed to expand to the desired area at which point compression was begun at a rate of $3 \text{ \AA}^2/\text{molecule}\cdot\text{min}$ until film collapse was observed. Between each experiment the trough and barriers were cleaned by two washings with a decontaminating solution (Extran 1000), followed by thorough rinses with EtOH and CHCl_3 . In addition, the Wilhelmy plate was cleaned by sonication for 90 minutes at 50°C in Extran 1000 solution.

Preparation of cytochrome b_5 .

The soluble fragment of rat liver cytochrome b_5 (wild type and a variant with a 6-histidine sequence fused to the C-terminus) was expressed in *Escherichia coli* strain TB-1 from synthetic genes generously provided by Dr. S. G. Sligar [81]. The cells were grown in LB broth with 200 mg/L ampicillin at 37°C in several inoculation steps. Cells were harvested by centrifugation and lysed in 50 mM Tris-HCl buffer, pH 8.0, with 1 mg/mL lysozyme, 20 $\mu\text{g/mL}$ DNase I and 10 $\mu\text{g/mL}$ RNase A. Cell debris was removed by centrifugation at $10,000 \times g$ for 30 minutes. Wild-type cytochrome b_5 was purified according to the procedures described by Beck von Bodman et al. [81]. For the 6-histidine variant, the supernatant was applied to a NTA-Nickel column (QIAGEN Inc.) equilibrated in 50 mM Tris-HCl, pH 8.0, with 20 mM imidazole. The column was extensively washed with 20 mM imidazole and the 6-His cyt b_5 was eluted with 250 mM imidazole. A final size exclusion chromatographic step (Sephacryl S-200 HR, Pharmacia; Uppsala, Sweden), eluting the protein in either 20 mM MOPS, 100 mM NaCl, pH 7.5 or labeling buffer (see below), gave protein homogenous by SDS-PAGE.

Fluorescent labeling of 6-His cyt b_5 .

Cyt b_5 was labeled with fluorescein succinimidyl ester (FSE; Molecular Probes, Inc., Eugene OR) or rhodamine succinimidyl ester (RSE) following the standard procedure [53] with a slight modification. Briefly, 10 mM FSE (or RSE) in dry DMF was added

dropwise, with stirring, to a solution of ca. 100 μM cyt b_5 (in 0.1 M NaHCO_3 , 0.1 M NaCl , pH 8.3) to yield a dye/protein ratio of 2:1. The reaction mixture was incubated at room temperature for one hour. According to the standard protocol, the reaction was quenched by the addition of hydroxylamine to a final concentration of 0.15 M. However, as described above, this step was found to negatively effect the affinity of protein for IDA-Cu surfaces. Therefore, this step was omitted. Labeled protein was separated from unreacted dye by gel filtration chromatography on pre-packed Sephadex G-25 columns (PD-10, Pharmacia, Uppsala, Sweden) pre-equilibrated with 20 mM MOPS, 100 mM NaCl , pH 7.5. Absorbance measurements using extinction coefficients of $130 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 413 nm and $68 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 495 nm for cyt b_5 and fluorescein, respectively, indicate a fluorescein/cyt b_5 ratio of approximately 1:1. The resulting labeled 6-His cyt b_5 behaves similarly to unlabeled 6-His cyt b_5 in analytical IMAC on an IDA-Cu support, which indicates that the labeling reaction does not interfere with the ability of the 6-His tail to interact with immobilized IDA-Cu.

Metal-affinity chromatography.

The IMAC column (TSK Chelate-G6000XL, 7.5 cm x 7.5 mm, Tosohaas) was regenerated, loaded with Cu^{2+} [44], and subsequently equilibrated with 10 mL of 50 mM sodium phosphate, 0.5 M NaCl , 100 mM imidazole at pH 7.8, followed by 10 mL of the same buffer containing 20 mM imidazole. Experiments were performed at a flow rate of 1 mL/min. After injecting protein (100 μL of 50 μM 6-His cyt b_5) the column was washed with 6 mL of buffer containing 20 mM imidazole. The protein was then eluted with a linear gradient of imidazole from 20 to 200 mM over 54 mL, and the eluent was monitored by absorbance at 413 nm (heme Soret band) to detect protein or at 495 nm detecting fluorescein. The chromatograms have been corrected for the changing baseline due to absorbance of imidazole by subtraction of the trace obtained in the absence of protein.

Monolayer studies of 6-His cyt b₅-PSIDA-Cu interactions.

Monolayer studies were carried out on a computer-controlled Langmuir film balance (KSV Instruments, Helsinki) with a surface area of 75 x 230 mm². The entire film balance was placed inside a Plexiglas box, and the temperature was controlled by a circulating water bath. The surface pressure was measured by the Wilhelmy plate method using plates cut from filter paper (Whatman, No. 1). The plate was rinsed thoroughly with chloroform and calibrated with stearic acid prior to use. Lipid solutions were prepared in chloroform, and IDA lipid/SOPC mixtures were prepared by mixing appropriate volumes of stock solutions prior to spreading. The IDA lipids were pre-metalated by adding an aliquot of concentrated CuCl₂ in methanol to the lipid solutions. Cu²⁺ excess in the lipid solution is ~10-15 %.

Protein binding experiments were carried out on subphase buffered with 20 mM MOPS, 100 mM NaCl, pH 7.5. Monolayers were compressed at a constant rate of 3 Å²/molecule·min. A 15-minute waiting period prior to compression allowed for solvent evaporation. Once the desired surface pressure was attained, the instrument was switched to constant-pressure mode, and the monolayer was allowed to equilibrate for 15-30 minutes. The desired amount of protein was injected into the subphase behind the barrier and mixed using a peristaltic pump which circulated the subphase from one side of the trough to the other. Changes in surface area were recorded as a function of time. After each experiment, the trough was thoroughly cleaned with detergent (RBS 35), Milli-Q water and chloroform. All measurements were performed at 25 °C.

Fluorescence microscopy.

The Langmuir instrument was placed above the stage of an inverted epifluorescence microscope (Zeiss Axiovert 35). A circular quartz window (~5 cm in diameter) was incorporated into the bottom of the trough. An HBO 100 W/2 Hg arc lamp was used as the excitation source. A long-working-distance reflecting objective (36x, NA 0.5, 8 mm

working distance; Ealing Electro-Optics, Holliston, MA) transmits light from the far UV to near IR, allowing UV excitation of the pyrene fluorophore. Excitation and emission wavelengths were selected using appropriate narrow-band interference filters. Images of fluorescein-labeled protein were obtained using excitation in the range 450-490 nm and a 515-565 nm band pass emission filter (Zeiss set 487910). Rhodamine-labeled protein was observed *via* excitation (560DF40) and emission (630DF30) interference filters and an appropriate dichroic mirror (600DRLP02) from Omega Optical, Inc. (Brattleboro, VT). Pyrene fluorescence was excited by selection of the 334-nm line of the Hg lamp *via* a narrow bandpass (10 nm FWHM; Melles Griot, Irvine, CA) interference filter. By the choice of appropriate filters, it is possible to selectively observe pyrene monomer and excimer signals (405DF35 and 470DF40, respectively; Omega Optical, Inc.). The images were obtained *via* a variable-gain image intensifier (Hamamatsu C2400-68) with a solid-state CCD camera (Hamamatsu C2400-60) and were recorded on a video cassette recorder. Fluorescence images were digitized on a Macintosh Quadra 660AV and were manipulated using the program NIH Image (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

His	N _ε area (Å ²) ^a	N _δ area (Å ²) ^a	total area (Å ²) ^a	accessibility ^b	pK _a ^c
24	0.0	0.0	0.0	0.00	6.3
36	1.8	6.2	8.0	0.27	7.8
48	5.3	9.3	14.6	0.49	5.5
64	0.0	0.0	0.0	0.00	NA
81	11	6.0	17	0.58	6.7
82	0.0	0.0	0.0	0.00	NA
93	0.0	0.0	0.0	0.00	NA
97	0.0	6.1	6.1	0.21	NA
113	8.5	9.1	17.6	0.60	5.4
116	12	0.0	12	0.41	6.7
119	0.0	6.6	6.6	0.23	6.4

^a Accessible areas were determined as a Connolly surface accessible to a spherical probe of radius 1.93 Å simulating IDA-Cu [82] using InsightII. ^b Accessibility is determined as the fraction of the N_ε and N_δ areas of methylimidazole, 29.7 Å². ^c The protonation constants are reported in [83].

Table 3.1. Surface accessible areas and protonation constants of histidine residues of sperm whale myoglobin (Brookhaven file 1mbw.pdb) [62].

His	N _ε area (Å ²) ^a	N _δ area (Å ²) ^a	total area (Å ²) ^a	accessibility ^b
15	0.0	3.3	3.3	0.11
26	1.7	9.0	11	0.36
39	-	0.0	0.0	0.00
63	-	0.0	0.0	0.00
80	12	0.0	12	0.41

^a Accessible areas were determined as a Connolly surface accessible to a spherical probe of radius 1.93 Å simulating IDA-Cu [82] using InsightII. ^b Accessibility is determined as the fraction of the N_ε and N_δ areas of methylimidazole, 29.7 Å².

Table 3.2. Surface accessible areas of histidine residues of the soluble fraction of rat liver cytochrome b₅ (Brookhaven file 3b5c.pdb) [70].

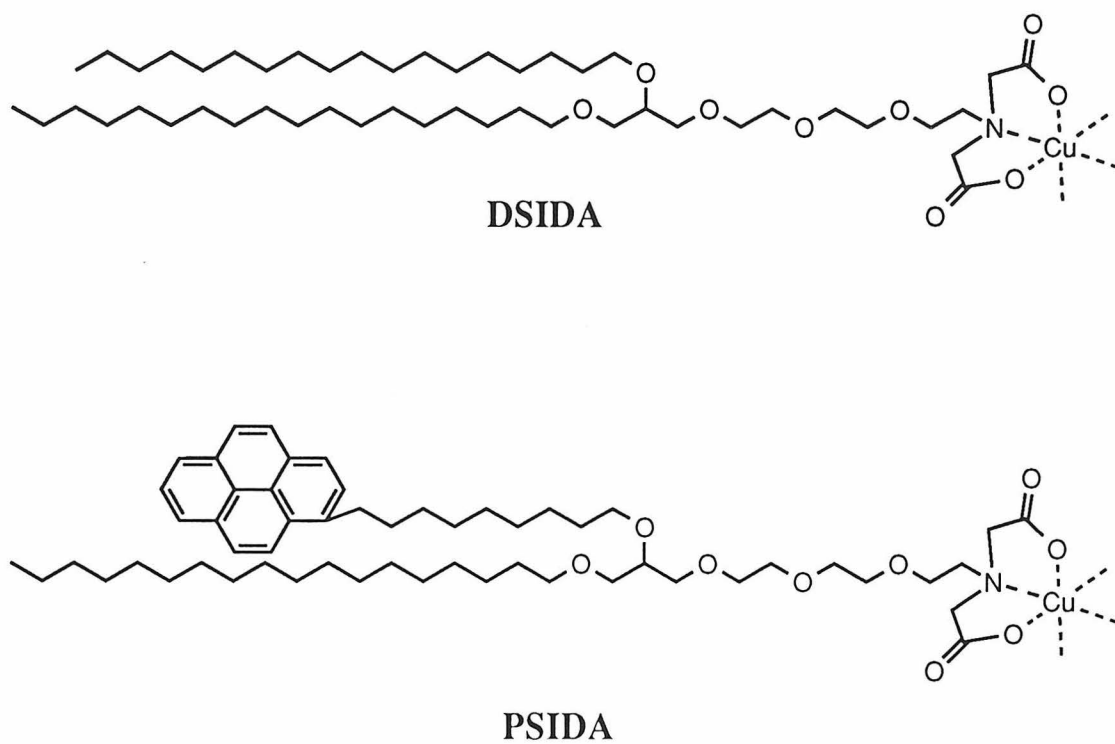


Figure 3.1. Structures of synthetic metal chelating lipids 1,2-distearyl-*rac*-glycero-3-(8-(3,6-dioxy)octyl)-1-amino-N,N-diacetic acid) (DSIDA) and 1-stearyl-2-(9-(1-pyrene)-nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl)-1-amino-N,N-diacetic acid) (PSIDA) shown as the copper complex.

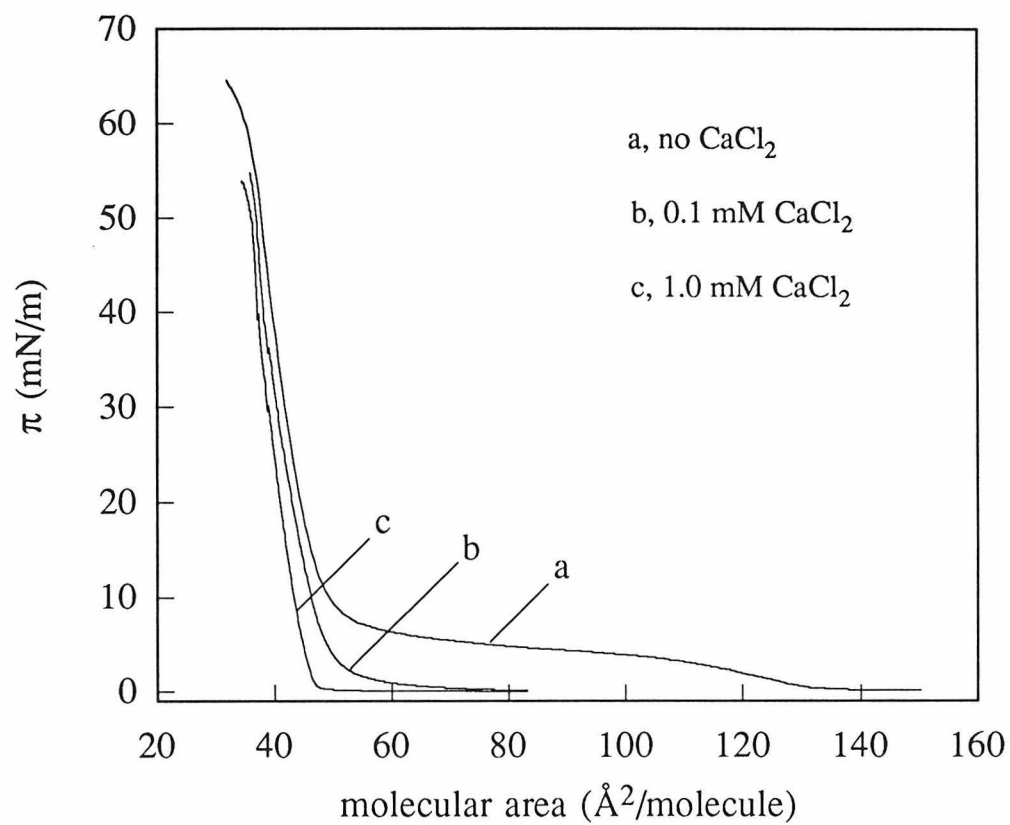


Figure 3.2. Surface pressure-molecular area isotherms of pure DSIDA lipid in the presence of varying amounts of CaCl_2 ; curve a, no divalent metal; b, 0.10 mM CaCl_2 ; c, 1.0 mM CaCl_2 on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C).

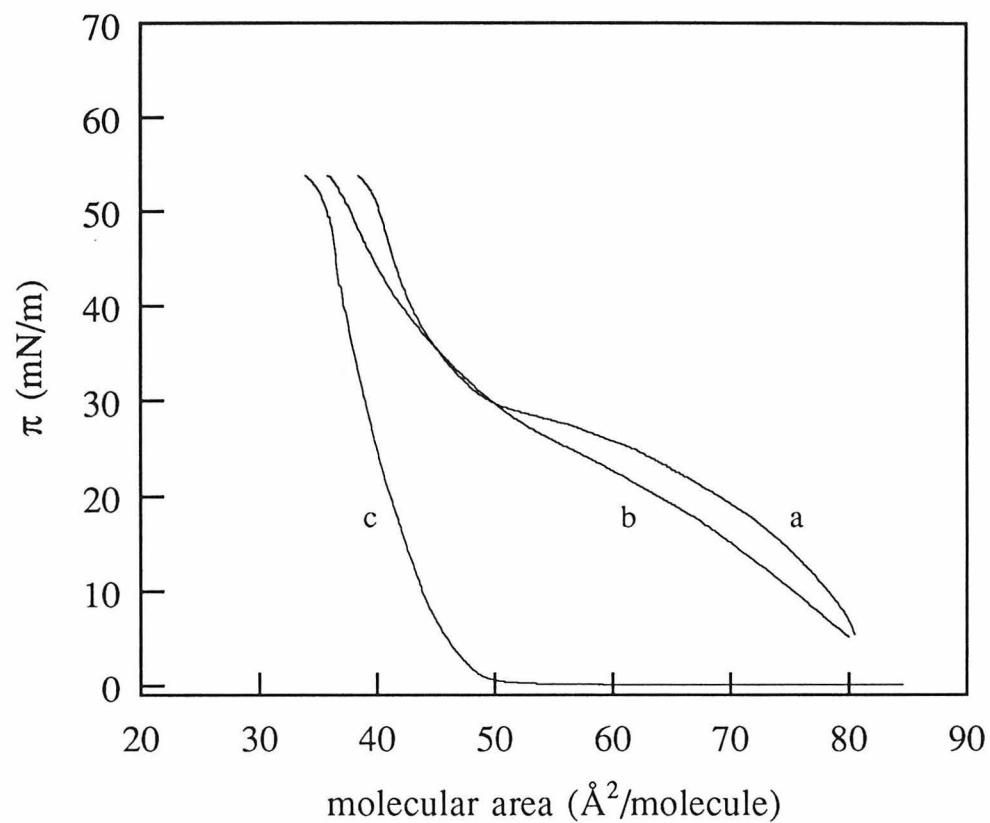


Figure 3.3. Surface pressure-molecular area isotherms of pure DSIDA lipid on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C) in the presence of: curve a, 10 μM CuCl_2 ; b, 1.0 mM CaCl_2 and 10 μM horse heart myoglobin; curve c, 10 μM CuCl_2 , 1.0 mM CaCl_2 and 10 μM horse heart myoglobin. Protein was injected into the subphase at 5 mN/m and the monolayer was allowed to expand to 80 $\text{\AA}^2/\text{molecule}$ before starting compression.

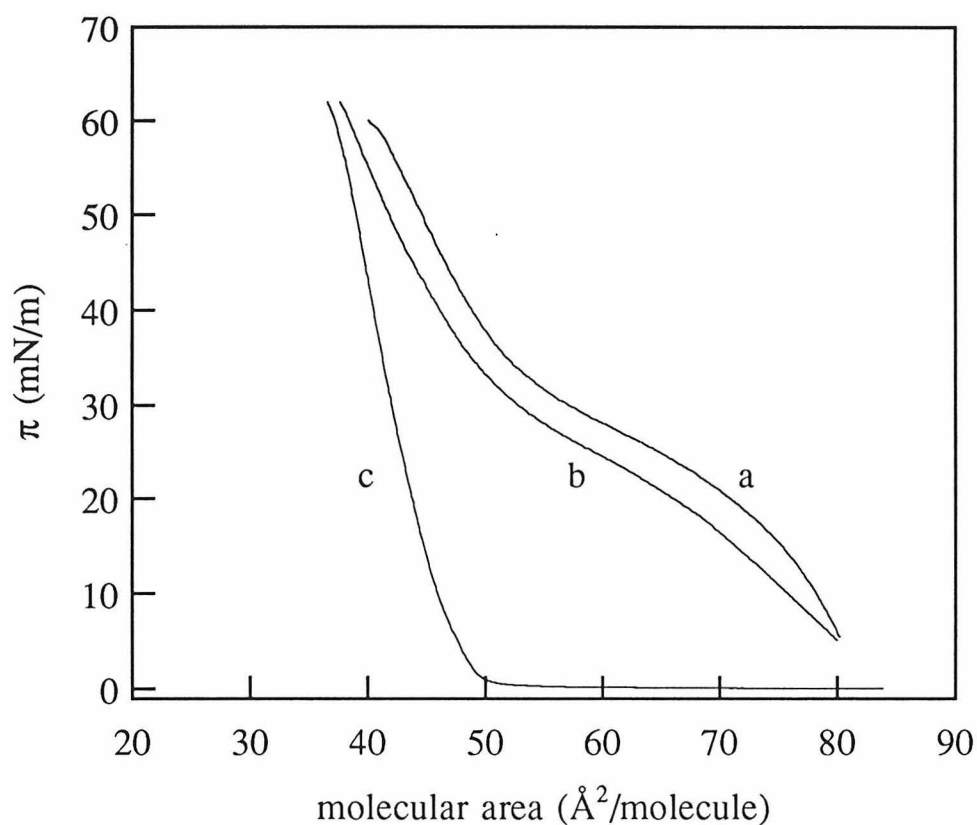


Figure 3.4. Surface pressure-molecular area isotherms of DSIDA/DSPC (1:19) mixed monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C) in the presence of: curve a, 10 μ M CuCl_2 ; b, 1.0 mM CaCl_2 and 10 μ M horse heart myoglobin; curve c, 10 μ M CuCl_2 , 1.0 mM CaCl_2 and 10 μ M horse heart myoglobin. Protein was injected into the subphase at 5 mN/m and the monolayer was allowed to expand to 80 $\text{\AA}^2/\text{molecule}$ before starting compression.

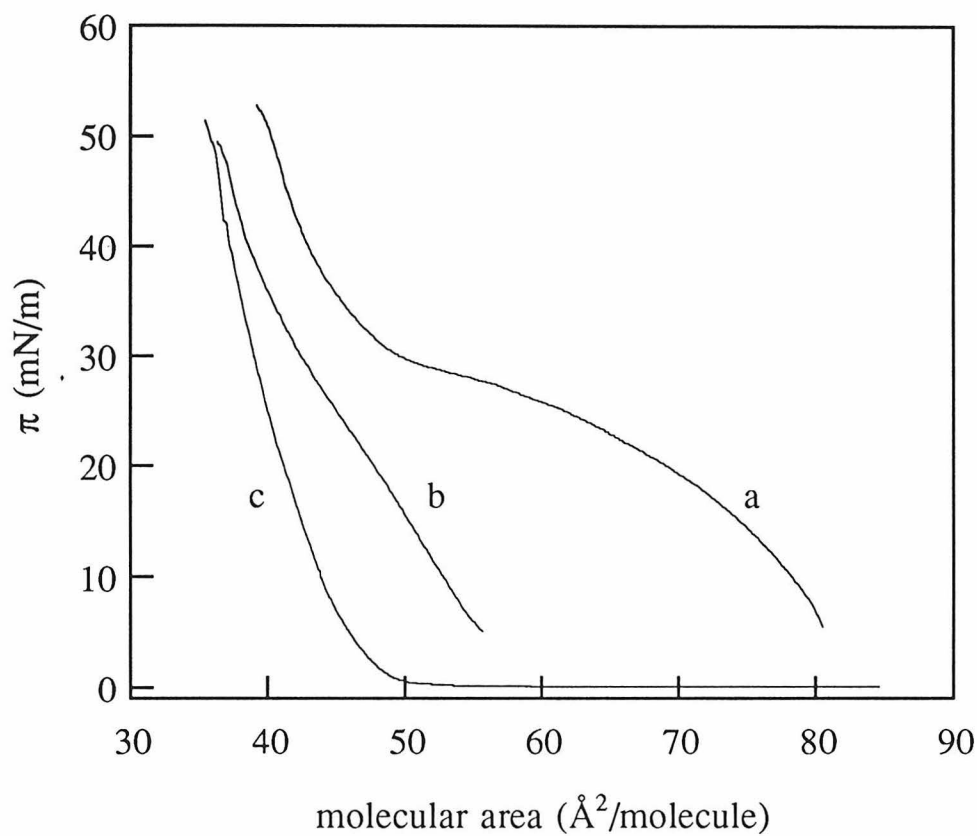


Figure 3.5. Surface pressure-molecular area isotherms of pure DSIDA lipid on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C) in the presence of: curve a, 10 μ M CuCl_2 ; b, 1.0 mM CaCl_2 and 10 μ M horse heart myoglobin; curve c, 10 μ M CuCl_2 , 1.0 mM CaCl_2 and 10 μ M horse heart myoglobin. Compression was begun $\sim$ 5 minutes after protein injection.

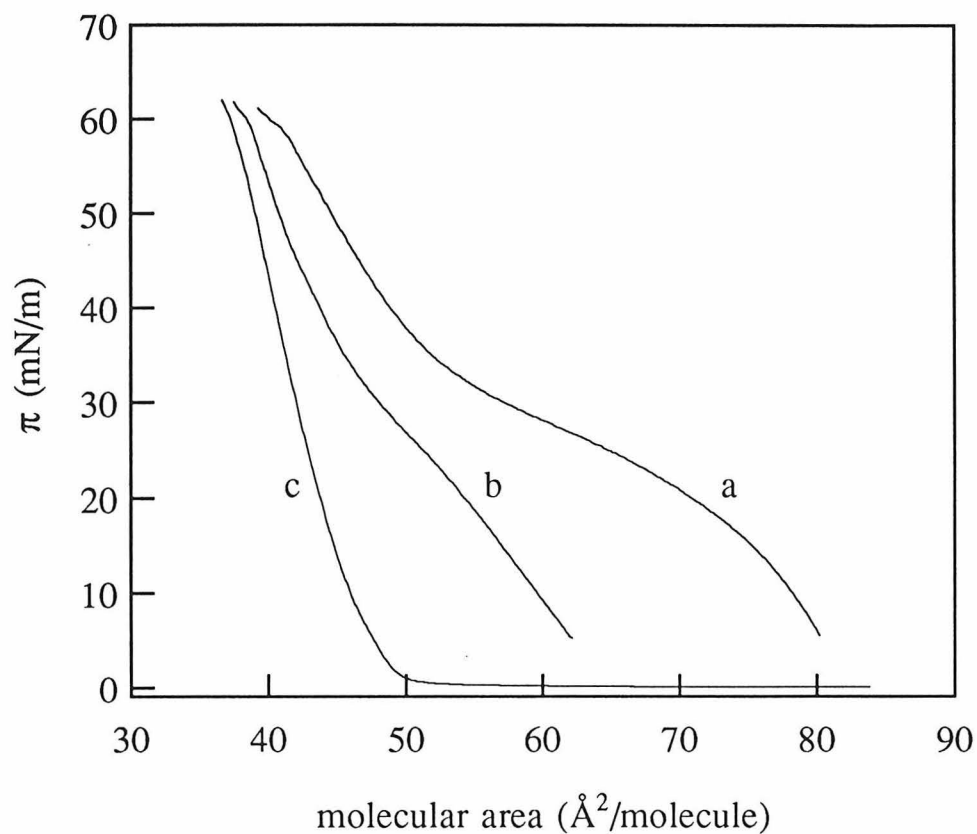


Figure 3.6. Surface pressure-molecular area isotherms of DSIDA/DSPC (1:19) mixed monolayers on buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5, 25 °C) in the presence of: curve a, 10 μ M CuCl_2 ; b, 1.0 mM CaCl_2 and 10 μ M horse heart myoglobin; curve c, 10 μ M CuCl_2 , 1.0 mM CaCl_2 and 10 μ M horse heart myoglobin. Compression was begun ~ 9 minutes after protein injection.

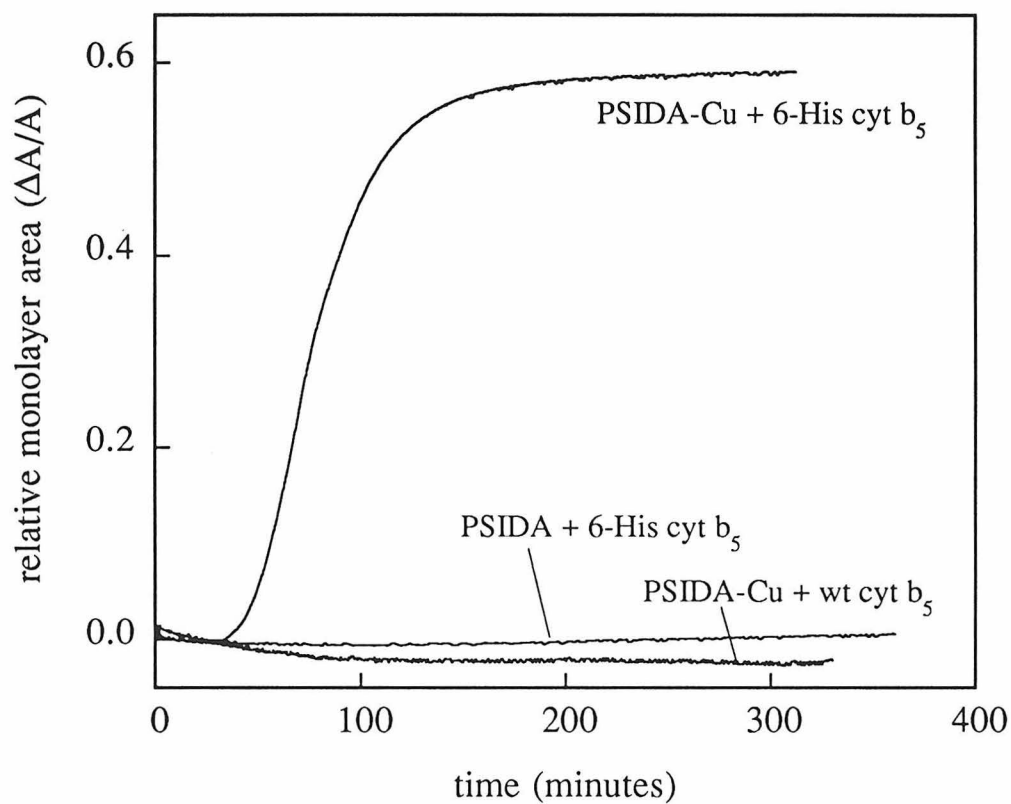


Figure 3.7. Relative monolayer area ($\Delta A/A$) versus time at $\pi = 25$ mN/m for PSIDA monolayers with 6-His cyt b_5 or wt cyt b_5 in the subphase as indicated. Protein was injected at $t = 0$ minutes. Initial protein concentration in the subphase is ~ 200 nM.

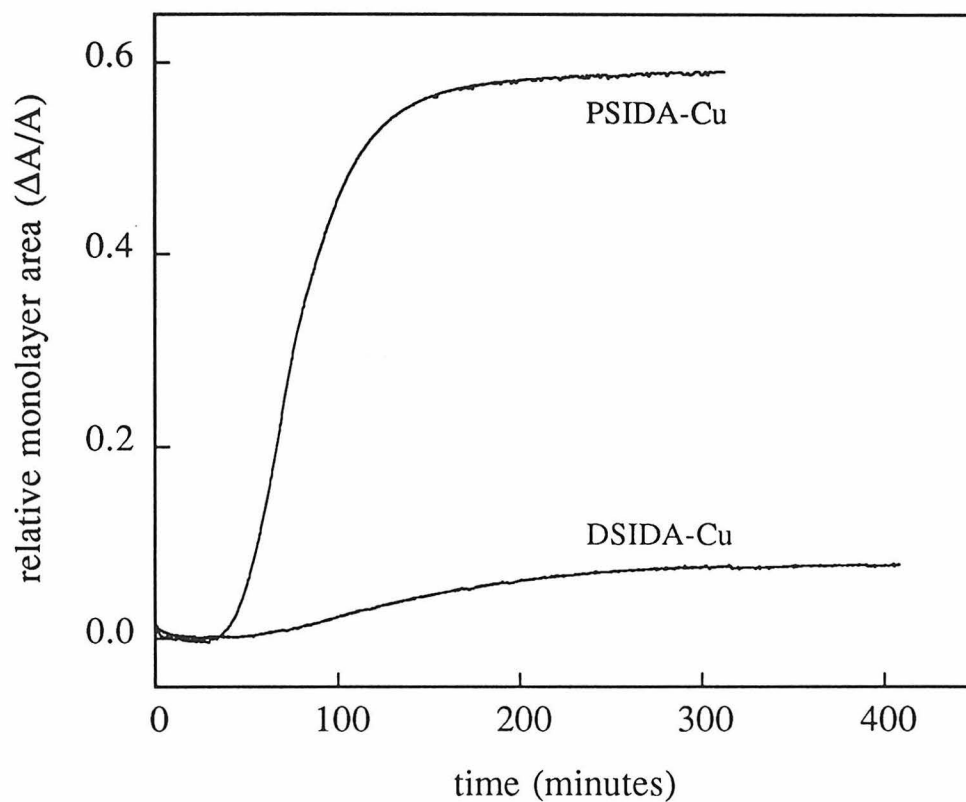


Figure 3.8. Relative monolayer area ($\Delta A/A$) versus time for DSIDA-Cu and PSIDA-Cu monolayers at $\pi = 25$ mN/m with 6-His cyt b_5 in the subphase. Protein was injected at $t = 0$ minutes. Initial protein concentration in the subphase is ~ 200 nM.

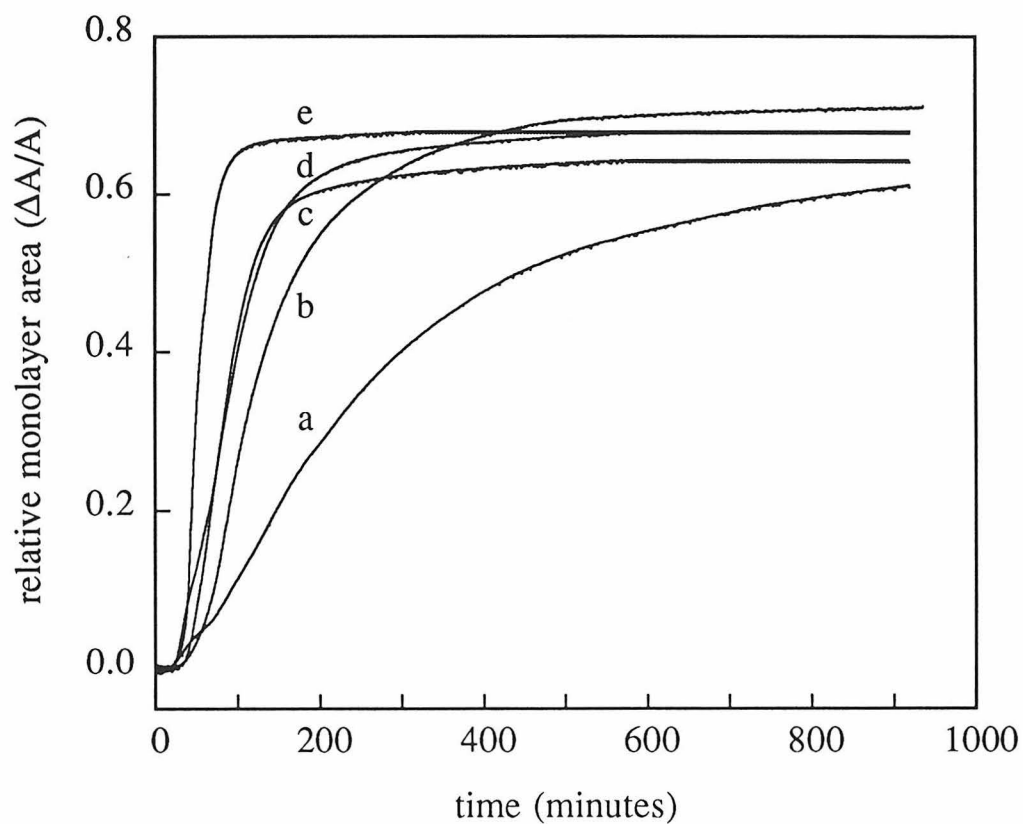


Figure 3.9. Relative monolayer area ($\Delta A/A$) versus time for pure PSIDA-Cu monolayers at $\pi = 25$ mN/m for varying 6-His cyt b_5 concentration in the subphase: curve a, 50 nM; b, 100 nM; c, 200 nM; d, 500 nM and e, 1000 nM. Protein was injected at $t = 0$ minutes.

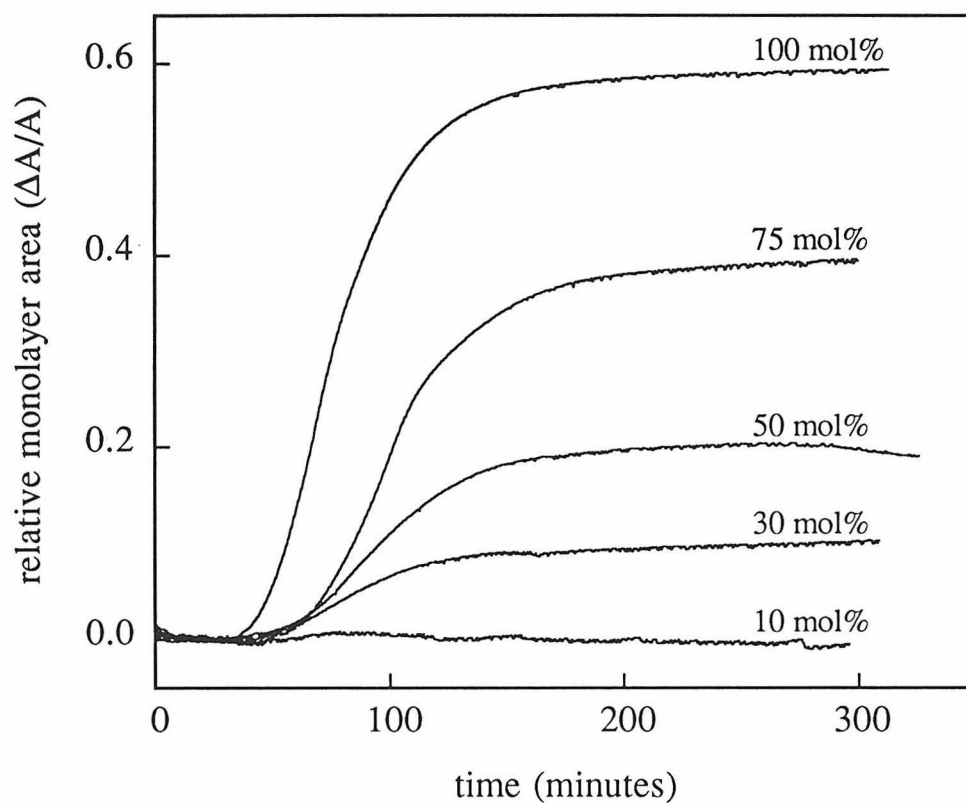


Figure 3.10. Relative monolayer area ($\Delta A/A$) versus time for mixed PSIDA-Cu/SOPC monolayers containing different mole fractions PSIDA as indicated. The measurements were carried out at 25 mN/m and subphase protein concentration of ~ 200 nM. Protein was injected at $t = 0$ minutes.

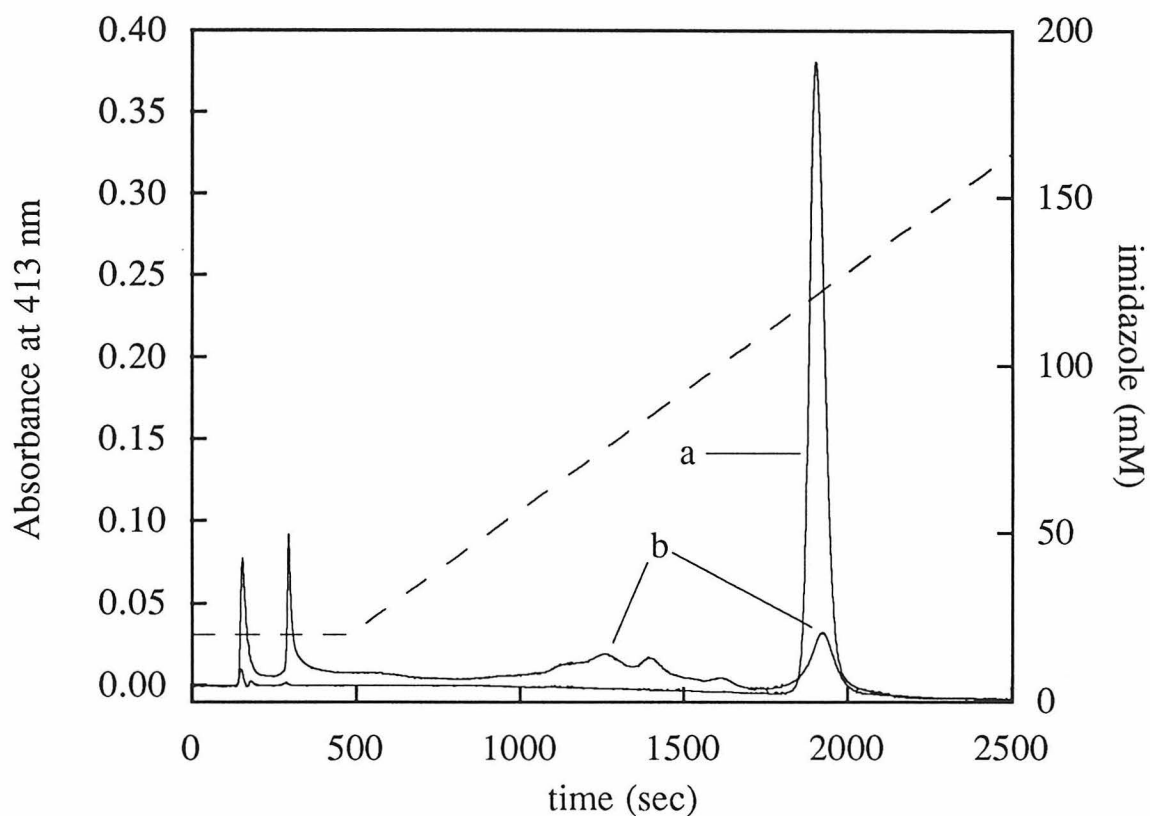


Figure 3.11. Chromatogram showing retention of 6-His cyt b_5 (a) and fluorescein-labeled 6-His cyt b_5 (b) in metal-affinity chromatography on an IDA-Cu-derivatized support. The protein was eluted with a linear gradient of imidazole (dotted line). Unlabeled protein is strongly retained and elutes as a single peak. The 6-His cyt b_5 was labeled according to the standard procedure including quenching of the reaction with hydroxylamine. Binding of the labeled protein is heterogeneous and weaker overall.

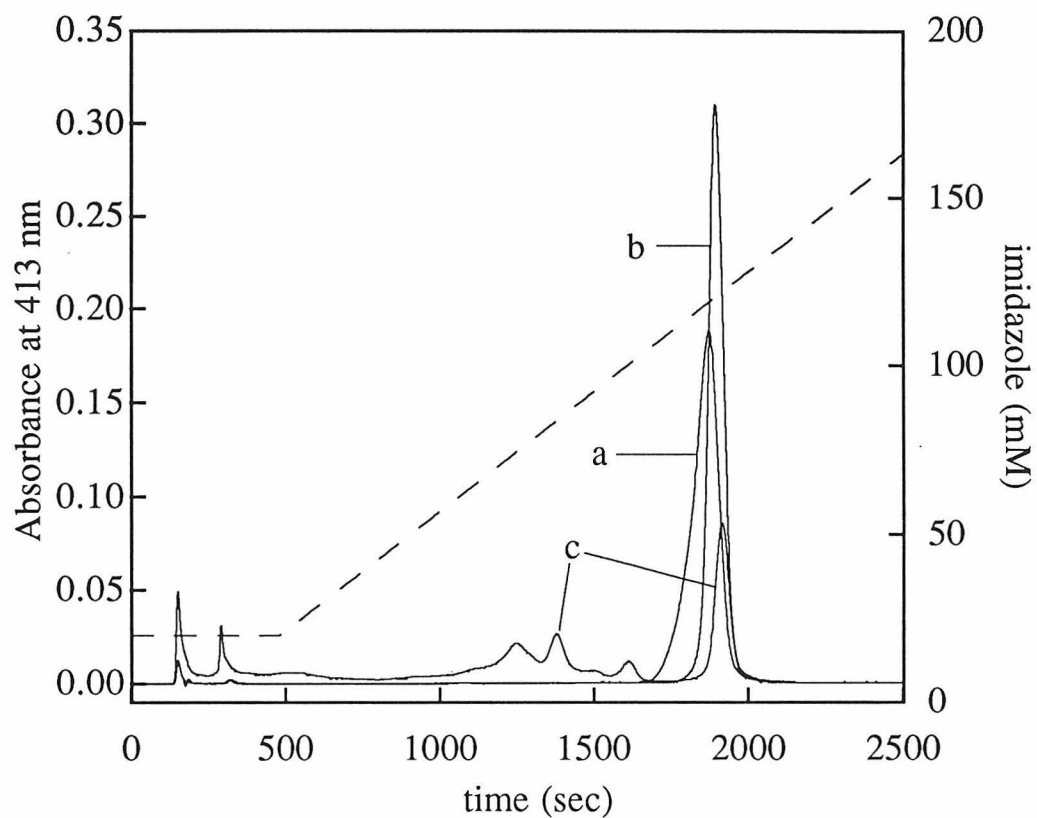


Figure 3.12. Chromatogram showing retention of 6-His cyt b_5 in metal-affinity chromatography on an IDA-Cu-derivatized support. The protein was eluted with a linear gradient of imidazole (dotted line). The protein was incubated for 1 hour in (a) pure buffer, (b) buffer with 6% DMF or (c) buffer with 0.15 M hydroxylamine.

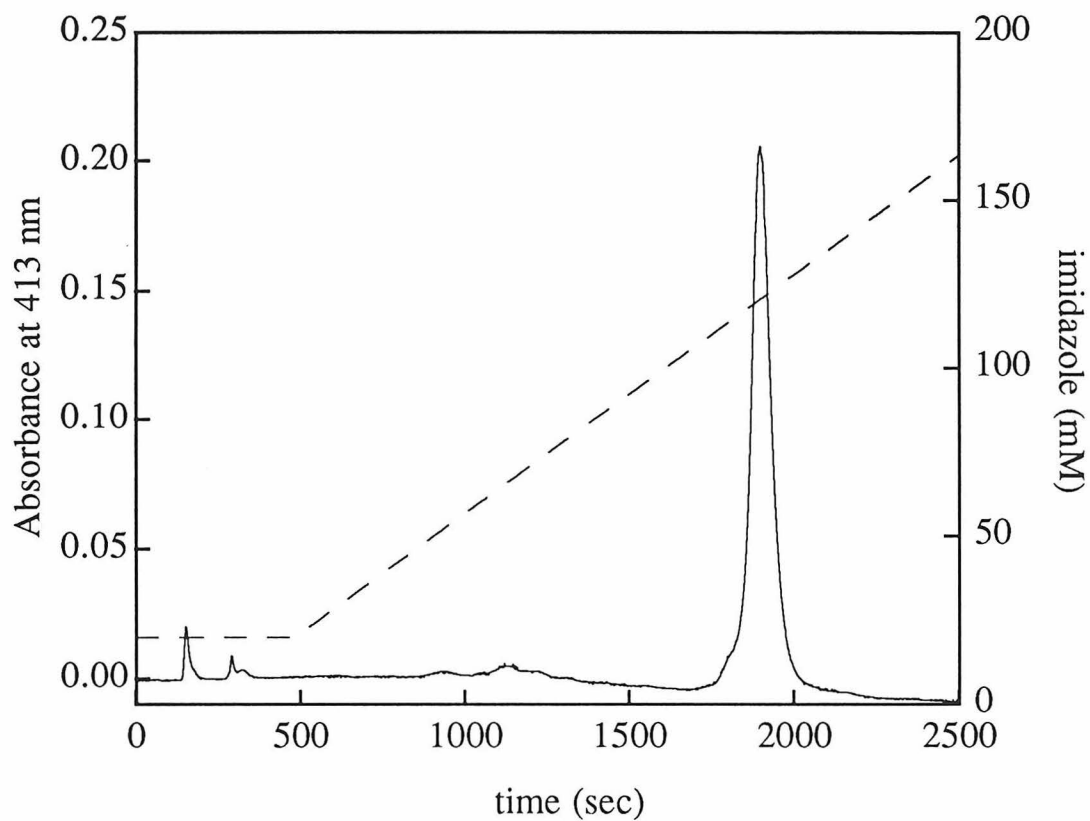


Figure 3.13. Chromatogram showing retention of 6-His cyt b_5 labeled with fluorescein succinimidyl ester according to the modified procedure in metal affinity chromatography on an IDA-Cu-derivatized support. Protein was eluted with a linear gradient of imidazole (dotted line). More than 80% of the protein elutes in a single peak at the same imidazole concentration as the unlabeled protein.

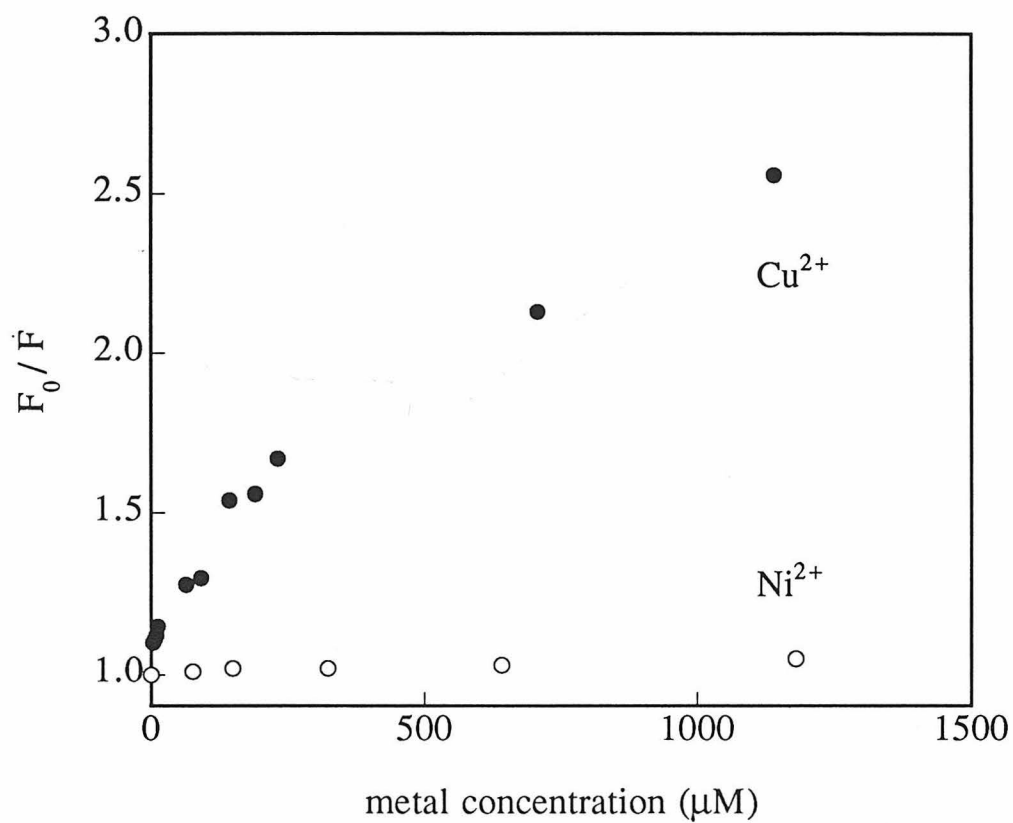


Figure 3.14. Stern-Volmer plot of quenching of fluorescein-labeled 6-His cyt b_5 by CuCl_2 and NiCl_2 in solution. The nonlinearity of F_0/F as a function of $[\text{Cu}^{2+}]$ indicates a combination of static and dynamic quenching. Cu^{2+} is clearly a much more efficient quencher of the fluorescein than is Ni^{2+} .

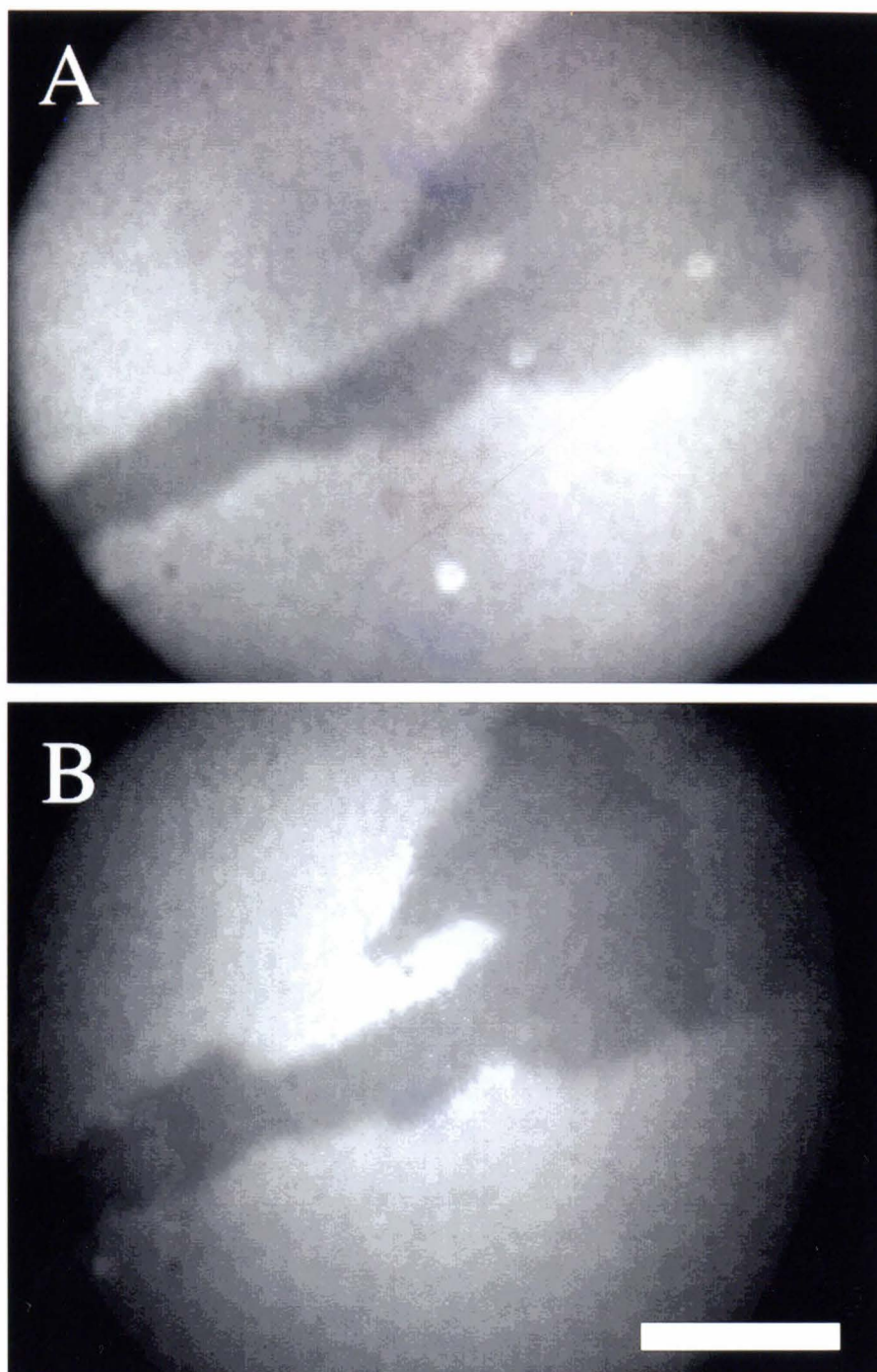


Figure 3.15. (A) Pyrene fluorescence micrograph of a supported monolayer of PSIDA-Ni/DBPC (1:1). (B) Rhodamine fluorescence micrograph of rhodamine-labeled 6-His cyt b₅ bound to the lipid monolayer. Scale bar = 100 μm .

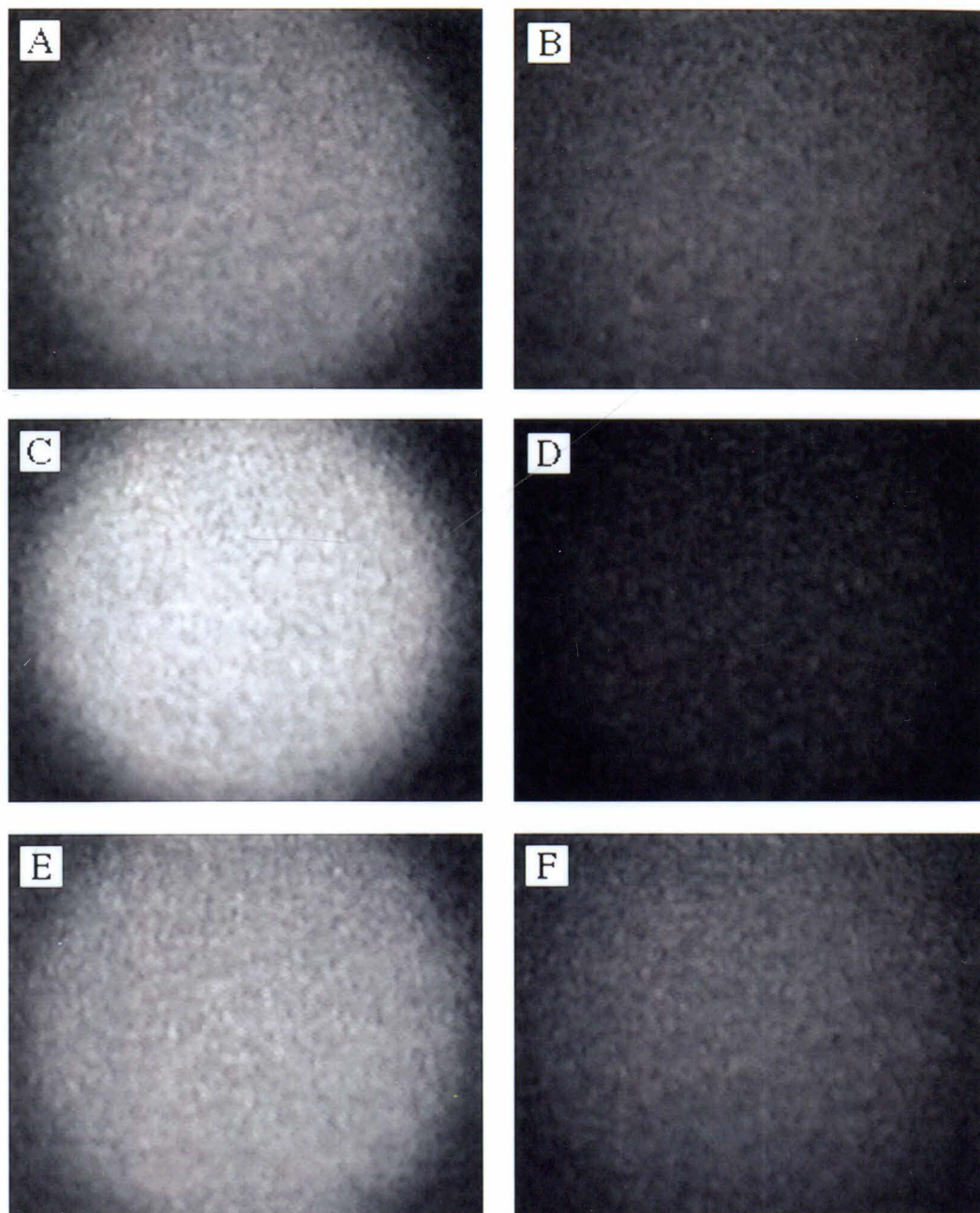


Figure 3.16. Fluorescence micrographs of fluorescein-labeled 6-His cyt b_5 in the presence of metal-chelating lipid monolayers spread on buffered subphase at $\pi = 25$ mN/m. (A) 10% PSIDA-Ni; (B) 10% PSIDA, no metal; (C) 30% PSIDA-Ni; (D) 30% PSIDA, no metal; (E) 100% PSIDA-Ni; (F) 100 % PSIDA, no metal.

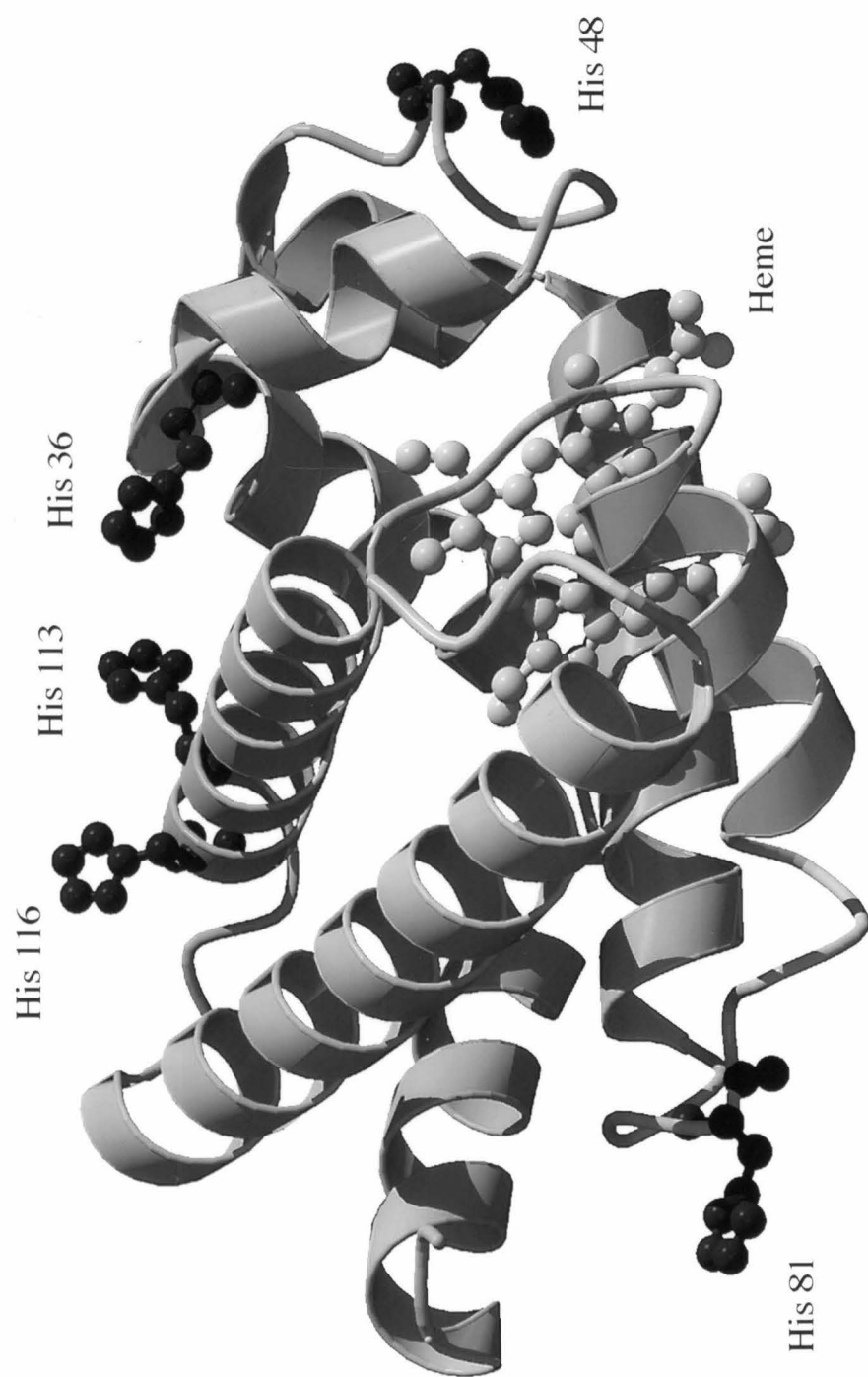


Figure 3.17. Three-dimensional x-ray crystal structure of sperm whale myoglobin showing the locations of the five surface-accessible histidines shared with horse heart myoglobin and the heme prosthetic group.

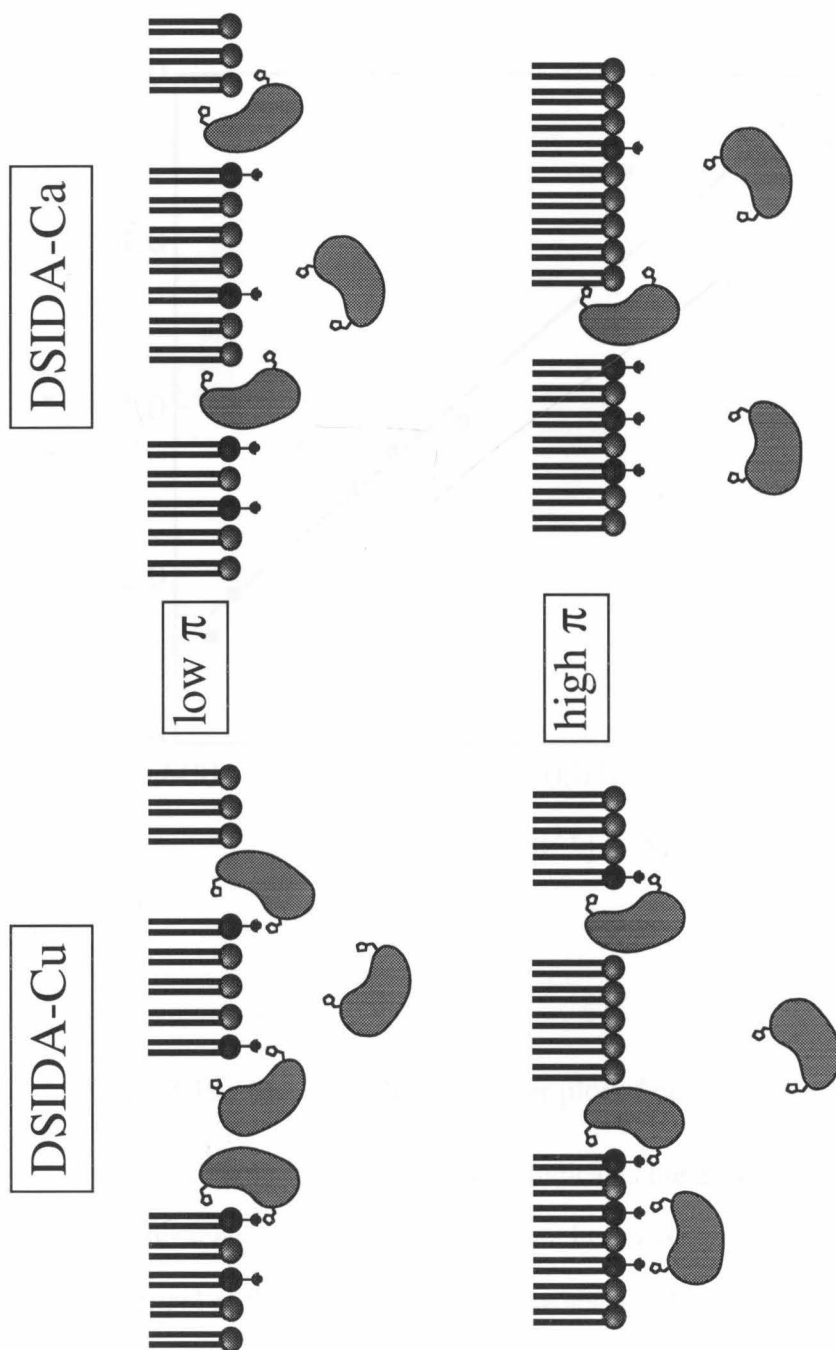


Figure 3.18. Schematic illustrating proposed model for interaction of myoglobin with DSIDA-Cu and DSIDA-Ca monolayers. At low pressure, protein penetrates both monolayers although the insertion is accompanied by coordination of histidines to the DSIDA-Cu monolayer. At high pressure, the majority of the protein is excluded from the interface. However, more protein remains irreversibly bound to the DSIDA-Cu monolayer due to the specific histidine-copper interaction.

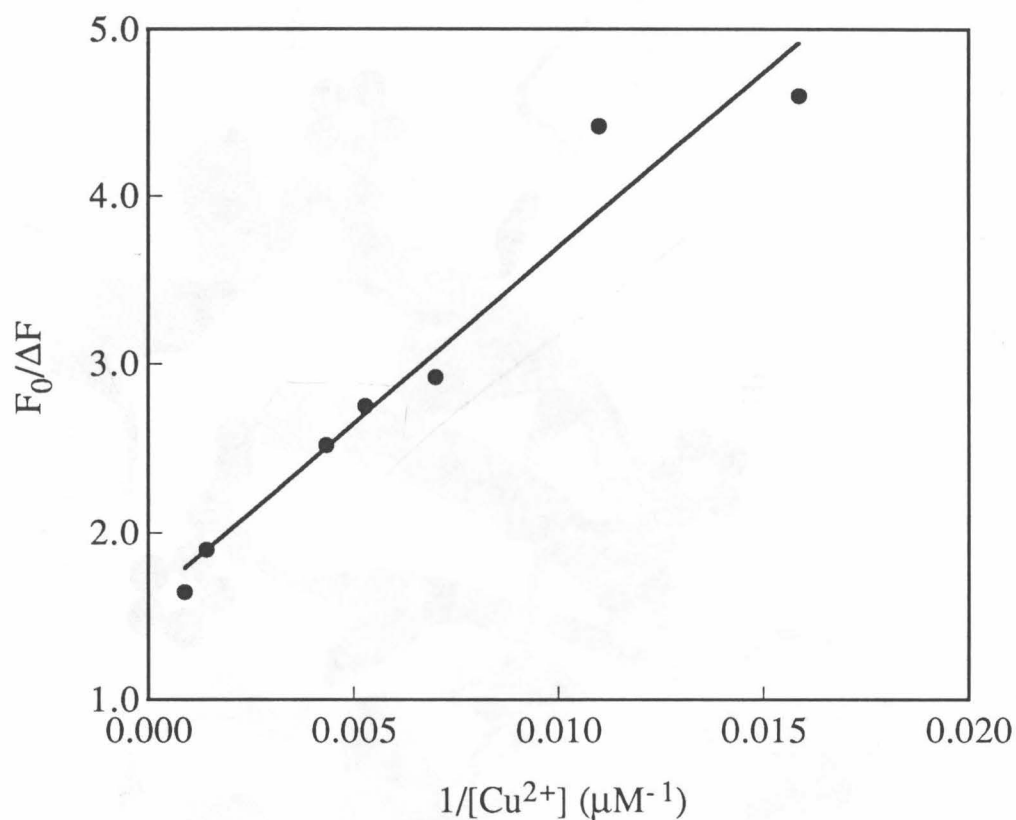


Figure 3.19. Modified Stern-Volmer plot of quenching of FI 6-His cyt b_5 by Cu^{2+} in solution. The linear fit indicates the existence of two populations of fluorescein, one accessible and one inaccessible to quenching. The reciprocal of the y-intercept gives the accessible fraction ($f_a = 0.63$) while the reciprocal of the slope is equal to $f_a K$, indicating $K = 130 \mu M^{-1}$ ($R = 0.97$).

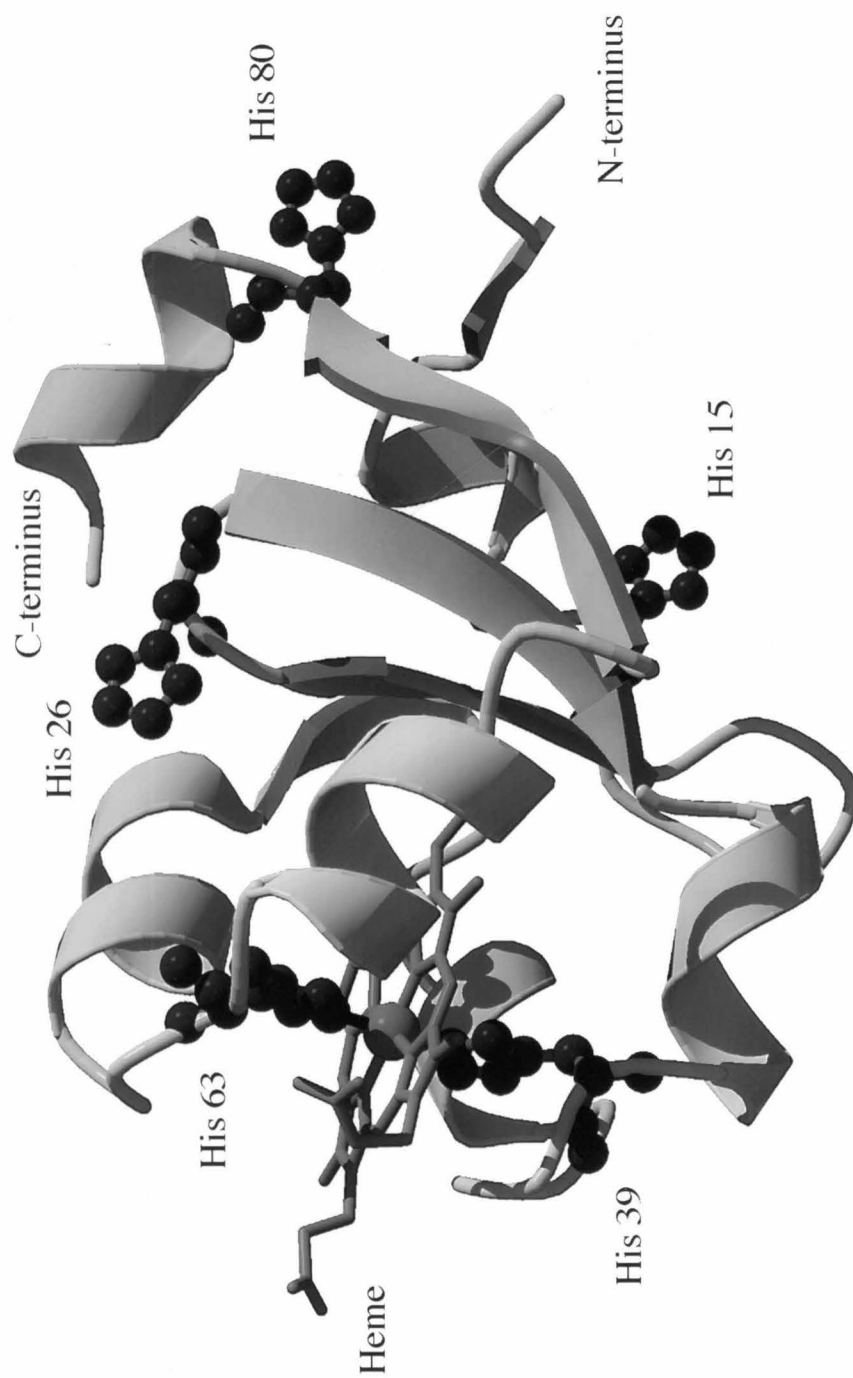


Figure 3.20. Three-dimensional x-ray crystal structure of rat liver cytochrome b₅ highlighting the relative locations of the protein's histidine residues, N- and C-termini and heme prosthetic group.

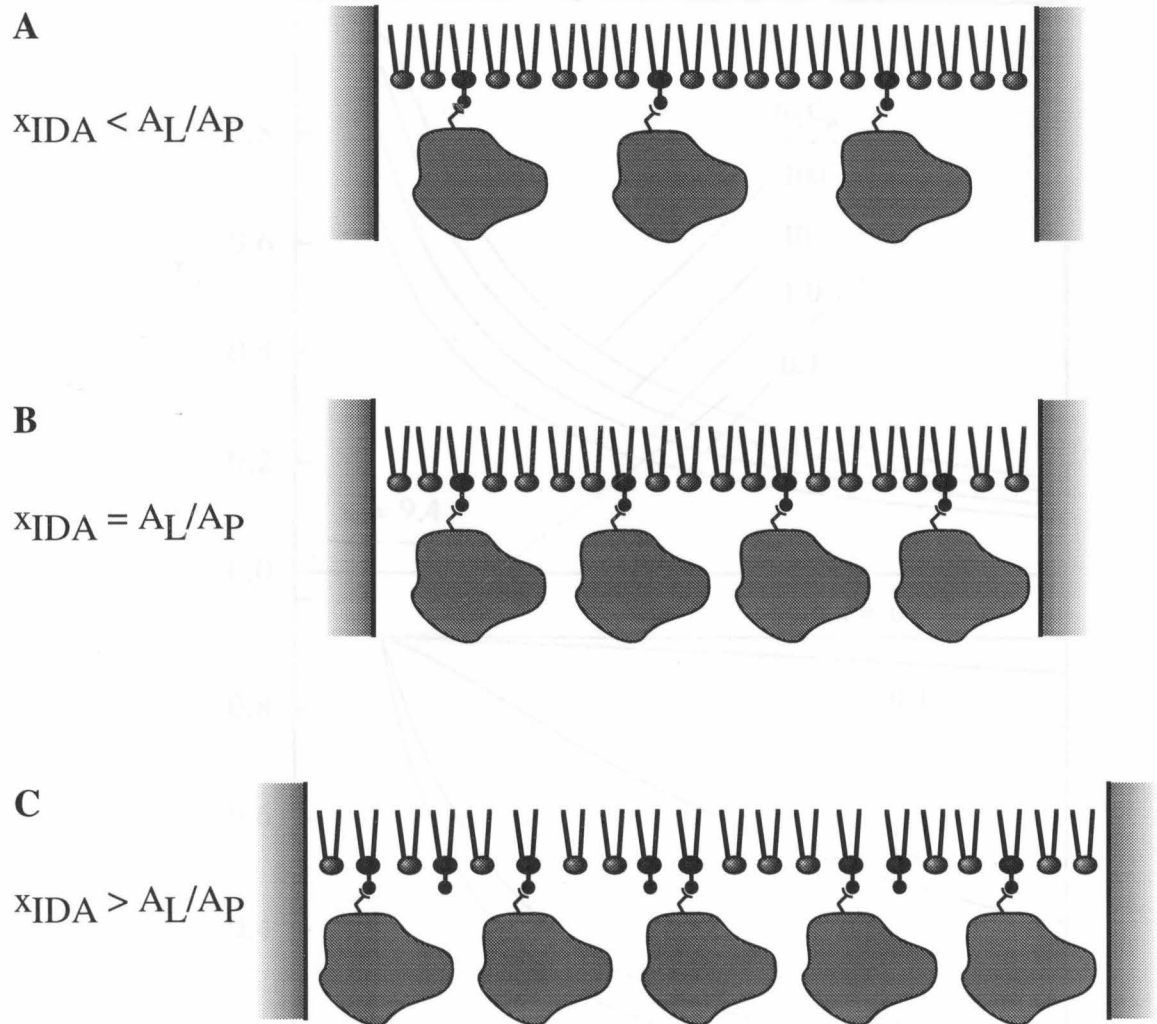


Figure 3.21. Proposed model for monolayer area increase due to protein binding.

(A) $x_{IDA} < A_L/A_P$. Protein binds IDA-Cu lipids without affecting monolayer surface area. (B) $x_{IDA} = A_L/A_P$. Bound protein forms a monolayer beneath lipid monolayer. Essentially all IDA-Cu binding sites are occupied, and no increase in area is observed. (C) $x_{IDA} > A_L/A_P$. Protein binding disrupts lipid packing and increases monolayer surface area at constant surface pressure. x_{IDA} = mole fraction IDA lipid in the monolayer; A_L = area occupied by one lipid; A_P = area occupied by one protein.

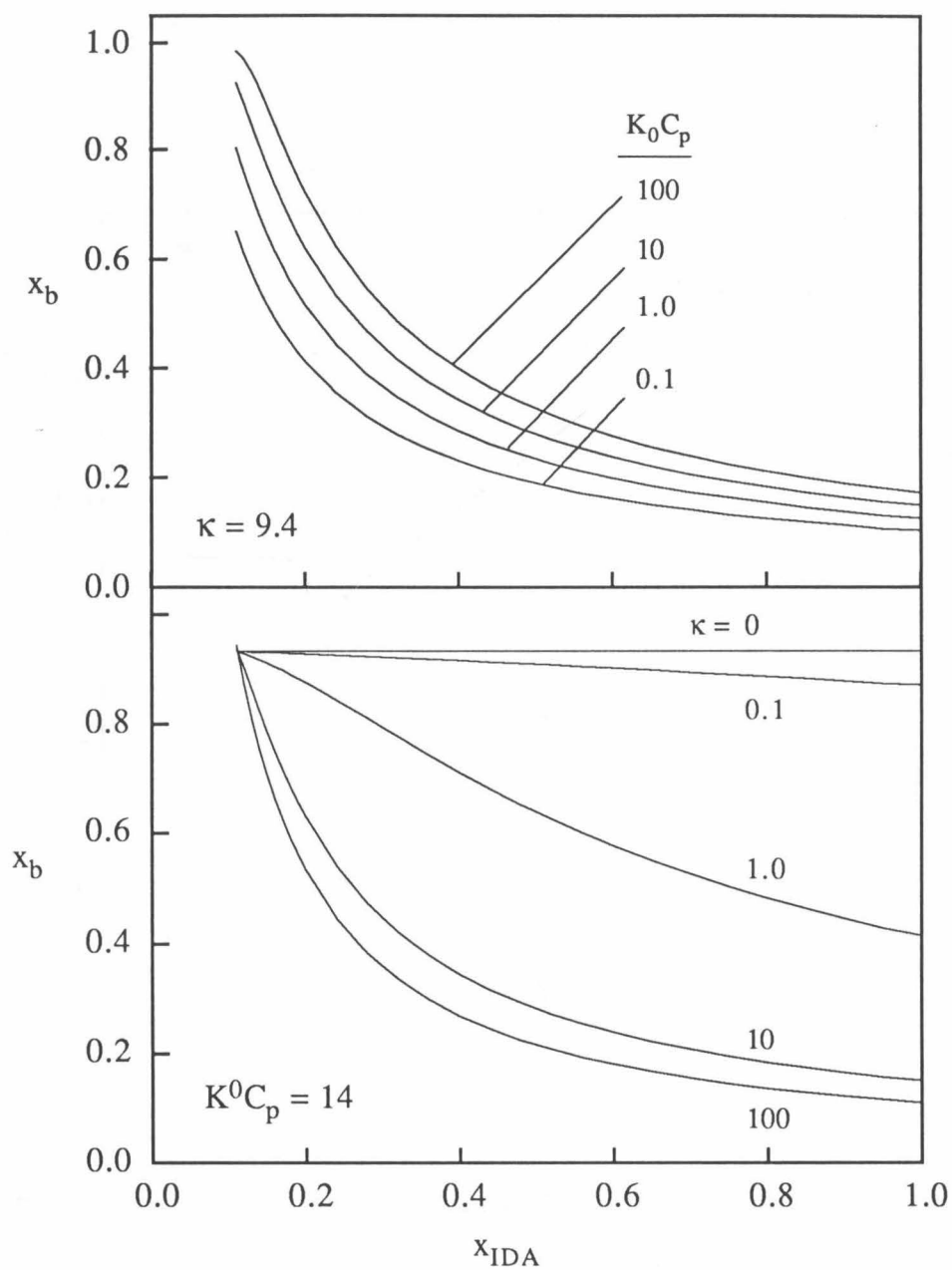


Figure 3.22. Predicted fraction of protein-bound IDA lipids, x_b , as a function of mole fraction IDA lipid in mixed monolayers. (a) Effect of binding strength ($\kappa = 9.4$). (b) Effect of interaction parameter, κ ($K_0C_p = 14$).

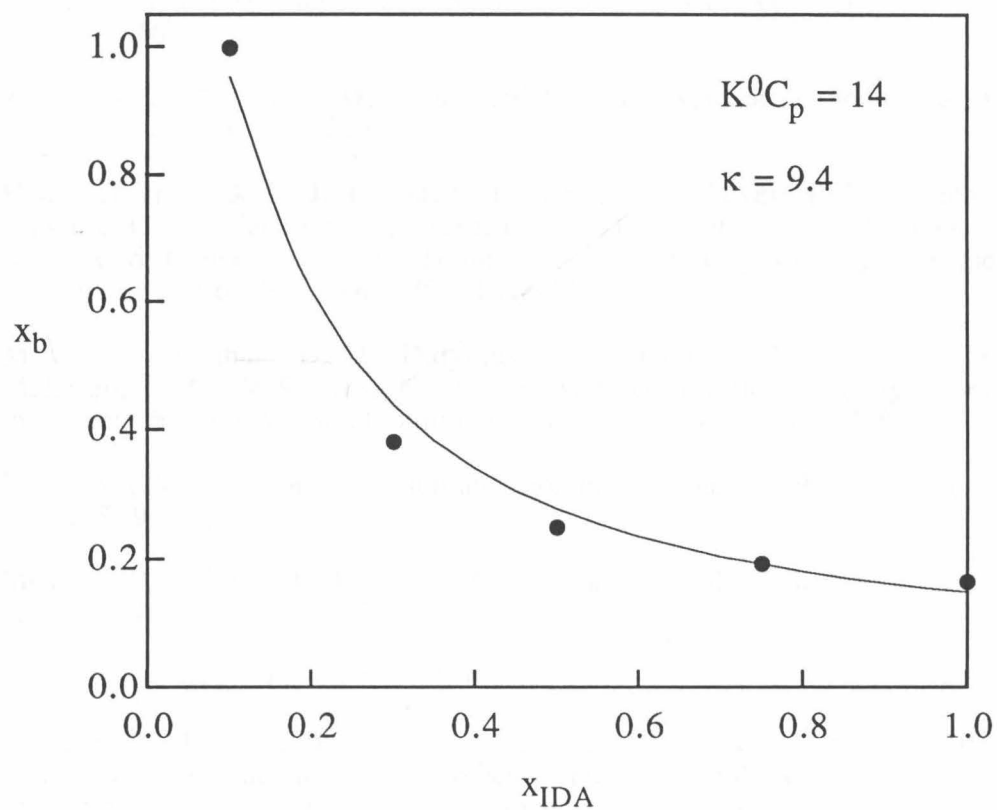


Figure 3.23. Fraction of protein-bound PSIDA-Cu lipids as a function of mole fraction IDA lipid in mixed monolayers with 200 nM 6-His cyt b_5 in the subphase (from Figure 3.10). Solid line represents least squares fit of the isotherm, equation (16). The binding constant, K^0 , determined from the fit was $7.8 \times 10^8 \text{ M}^{-1}$, and the interaction parameter, κ , was found to be 9.4.

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Chapter 4

**Two-dimensional crystallization of streptavidin on
metal-chelating lipid monolayers.**

Preface

Parts of the following chapter are adapted from a previously published work. The experiments concerning Brewster angle microscopy of streptavidin crystals on DOIDA-Cu monolayers were included in W. Frey, W.R. Schief, D.W. Pack, C.-T. Chen, A. Chilkoti, P.S. Stayton, V. Vogel and F.H. Arnold, "Two-dimensional protein crystallization via metal-ion coordination by naturally occurring surface histidines," *Proc. Natl. Acad. Sci. USA* **93**, 4937-4941, 1996 (©1996 National Academy of Sciences).

This work was performed in collaboration with the group of Dr. Viola Vogel at the University of Washington at Seattle (UW). The experiments were designed by Dr. Wolfgang Frey, Bill Schief and the author. Brewster angle microscopy of streptavidin crystals grown on DOIDA-Cu monolayers was performed by Dr. Frey and Mr. Schief (UW). Brewster angle microscopy of streptavidin crystals grown on PSIDA-Cu monolayers was performed jointly by the author, Dr. Frey and Mr. Schief (UW). All fluorescence microscopy of streptavidin crystals was performed at Caltech by the author (with the exception of the polarization microscopy images which were obtained with Dr. Kevin M. Maloney).

Introduction

Living cells are capable of utilizing their inherently ordered cellular membranes as a template to direct the growth of organized materials. One example of this process is the growth of two-dimensional (2D) protein or glycoprotein crystals (S-layers) which form the outermost layer of a very wide variety of both gram-positive and gram-negative bacteria in nature [1, 2]. In their natural environments, S-layers function as structure maintaining elements, protective coats, molecular sieves, and recognition elements. However, S-layers may also be employed as new materials for the construction of molecular filters with steep cut-off for exclusion [3], patterned immobilization and affinity matrices [4], biosensors [5], and patterning masks for nanolithography [6].

It has been known for a number of years that it is possible to mimic these ordered protein materials by growth of 2D protein crystals on Langmuir monolayers [7, 8]. The ability to crystallize proteins in two dimensions leads to applications in such diverse fields as structural biology and materials science. Perhaps the most obvious application is the use of 2D protein crystals for structure determination by electron diffraction [9, 10]. In fact, recent technological developments have allowed near-atomic resolution structures to be obtained from 2D streptavidin crystals [11]. Furthermore, 2D protein crystals may be useful for seeding the epitaxial growth of three-dimensional protein crystals, and may allow the crystallization of proteins which are difficult to crystallize by other methods [12, 13].

In general, 2D protein crystallization requires the immobilization and orientation of protein at the monolayer surface in high concentration, without loss of mobility. The binding and orientation of proteins to lipid monolayers are best attained by utilizing a lipid in which the hydrophilic headgroup comprises a high affinity ligand for the protein of interest. However, non-specific electrostatic interactions and metal ion coordination (*vide infra*) have also been employed. Electrostatic interactions may be successful when the surface charge on the protein is asymmetric, promoting adsorption with a specific

orientation, but this approach is not general. Lateral and rotational mobility of the bound protein are required to allow formation of the optimum intermolecular contacts and efficient packing of the proteins within the crystal. This mobility is supplied largely by the properties of the lipid monolayer, and crystallization is generally performed in fluid-phase monolayers.

To date, a significant number of proteins and peptides have been crystallized in two dimensions. A non-comprehensive list of such proteins and the interactions used for targeting to the lipid monolayer is presented in Table 4.1. In more than half the cases listed, naturally occurring, specific ligands were used to promote crystallization of antibodies, enzymes, polypeptide toxins and other proteins (e.g. streptavidin, annexin VI). In each instance, a unique lipid containing the appropriate affinity ligand was required, and in many cases a novel synthesis of this lipid was necessary. The difficulty of obtaining the appropriate affinity lipid has likely slowed research in this area. Furthermore, for many potentially interesting proteins no convenient affinity ligand is available or even known.

Targeting of proteins to metal-chelating lipid surfaces can be attained through coordination of surface histidines to lipid-bound metal ions [14-17] (c.f. Chapter 3). At neutral pH, histidine side chains are primarily responsible for the affinity of metal ions for protein surfaces (e.g., $K_a \sim 10^{3.5} \text{ M}^{-1}$ for a single histidine interaction with IDA-Cu); at slightly basic pH, other groups such as lysine and the N-terminus can contribute to metal binding, as well [18]. Thus, this interaction should be amenable to 2D crystallization studies of any of the many proteins displaying surface histidines. Metal ion coordination to NTA-Ni lipids has recently been utilized by Kubalek *et al.* for the 2D crystallization of a poly(histidine) tagged, HIV-1 reverse transcriptase [19]. This approach may represent a general route to 2D crystallization of *recombinant* proteins, but is limited to those proteins which may be successfully expressed with the polyhistidine sequence. In contrast to NTA-Ni lipids, IDA-Cu lipids can effectively target proteins *via* coordination of naturally occurring histidines [14]. Thus, with IDA-Cu lipids, this approach is extended to any

protein containing solvent-accessible histidine side chains, either natural or genetically engineered. A single metal-chelating lipid could be used for many different proteins, including those for which no other affinity interaction may be available.

Here the 2D crystallization of streptavidin on IDA-Cu lipid monolayers is demonstrated. Streptavidin is a tetrameric protein best known for its ability to very tightly bind four equivalents of the small molecule biotin ($K_a \sim 10^{15} \text{ M}^{-1}$) [20]. Streptavidin has previously been crystallized in two dimensions on Langmuir monolayers of biotin-containing lipids [21] and on monolayers of poly(1-benzyl-L-histidine) *via* non-specific electrostatic interactions [22]. In addition, the three-dimensional crystal structure of this protein is known [23, 24]. Streptavidin contains two surface-accessible histidine side chains on each subunit at positions 87 and 127 (Figure 4.1). Using immobilized metal affinity chromatography (IMAC), streptavidin is shown to bind immobilized IDA-Cu *via* these histidine residues. Finally, streptavidin is observed to bind to and crystallize on IDA-Cu lipid monolayers in a copper-dependent fashion. This study therefore represents the first demonstration of a potentially *general* route to 2D crystallization of proteins.

Results

The lipids utilized for two-dimensional protein crystallization, 1,2-dioleoyl-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid) (DOIDA) [25] and 1-octadecyl-2-(9-(1-pyrene)nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid) (PSIDA) [15] (Figure 4.2), were described in detail in Chapter 2. The IDA headgroup and long ($\sim 11.5 \text{ \AA}$) headgroup spacer of these lipids allow the metal ion to be displayed in the aqueous subphase with coordination sites available for ligand binding, as discussed in Chapter 3. The unsaturated tails of DOIDA ensure the stability and fluidity of Langmuir monolayers of this lipid. As shown in Figure 2.6, DOIDA forms stable monolayers at the air-buffer interface, both in the presence and absence of metal ions, and remains in the fluid state at all surface pressures. Similarly, the bulky pyrene moiety of PSIDA results in fluid

Langmuir monolayers over a wide range of surface pressures, with and without metal, as shown in Figure 2.7. For protein crystallization, the chelating lipids were loaded with Cu^{2+} prior to spreading the monolayers, as Cu^{2+} in solution was found to inhibit binding and crystallization of streptavidin even under a biotinylated lipid monolayer [25].

The surface adsorption and crystallization of streptavidin was visualized using epifluorescence microscopy and Brewster angle microscopy (BAM). (BAM is a recently developed optical microscopy technique that allows imaging of molecular monolayers based on differences in optical thickness [26-28].) In each experiment, core streptavidin was injected beneath the IDA-Cu lipid monolayer at a surface pressure of 3-4 mN/m to a final protein concentration in the range 20-150 nM. Higher surface pressures were found to inhibit streptavidin crystallization, and crystals grown at 3 mN/m were disrupted upon compression of the monolayer to 15 to 20 mN/m [29]. It should be noted that all crystallization experiments with the DOIDA-Cu lipid were performed under an inert Ar atmosphere to prevent oxidation of the di-unsaturated tails during the often long time period (~4 hours) of the crystallization. Experiments with PSIDA-Cu were performed under Ar atmosphere as well. However, in this case the inert atmosphere was necessary to prevent oxygen quenching of the pyrene fluorescence.

Streptavidin crystallization on DOIDA-Cu monolayers.

A time sequence of BAM images demonstrating the aggregation process of streptavidin on a DOIDA-Cu monolayer is shown in Figure 4.3. In this experiment, the first aggregates appeared 15 minutes after protein was injected. The protein aggregates appeared bright on the dark background of the lipid monolayer, and a gray scale analysis of the images revealed that the protein density in the phase surrounding the aggregates was negligible. The earliest observed aggregates were irregular in shape. At longer times, however, the aggregates took on a rectangular shape and displayed sharp edges. The size of the aggregates increased with time whereas the rectangular shape was preserved. The

“tiles” tended to interlock at the corners or on the flat sides, again without a change in shape. However, for very large tiles ($>100 \times 100 \mu\text{m}^2$) the rectangular shape was often lost, and they became more pointed at the corners with angles significantly less than 90° .

The time course of crystallization was similarly visualized using fluorescence microscopy of rhodamine-labeled streptavidin (Figure 4.4). The kinetics of binding and crystallization in this experiment are apparently very different from the previously described experiment. These two experiments were performed with different experimental set-ups and using different streptavidin preparations. Therefore, the different kinetics are not surprising. Nevertheless, the process of adsorption and aggregation observed with fluorescence microscopy was very similar to that observed using BAM. Initially, very small, irregularly shaped aggregates were observed which began to take on the characteristic rectangular shape shortly thereafter. The tiles continued to grow in size over approximately four hours, maintaining the rectangular shape at all times. Finally, the tiles became quite large ($\sim 50 \times 50 \mu\text{m}^2$), covering most of the monolayer area. At this point, the tiles ceased to grow, and the monolayer became very stiff as evidenced by its resistance to flow. Streptavidin tiles of all sizes tended to associate with one another, as groups of tiles were much more common than individual tiles. However, the sharp edges and square corners were maintained, even in very dense groupings of large tiles. It should be noted that the relatively bright background in these images is due to free, out-of-focus protein in solution through which the excitation and emission light must pass, due to the inverted microscope configuration.

As mentioned above, streptavidin contains eight surface-accessible histidines; two per subunit. We wished to investigate which of these histidine side chains were responsible for coordination to the IDA-Cu lipid monolayers. Therefore, mutant proteins were investigated in which each of the two histidines, His 87 or His 127, has been replaced with a non-binding amino acid [25]. A variant in which His 127 is replaced by cysteine (H127C) was prepared previously [30]. H127C showed the same adsorption and

crystallization behavior as native streptavidin, indicating His 127 is not required for IDA-Cu monolayer binding. In contrast, when His 87 was replaced by alanine (H87A), the mutant protein showed no binding to a DOIDA-Cu monolayer over a period of 3 hours, as confirmed by a gray scale analysis of BAM images. Thus, two His 87 side chains on one face of the protein are the likely binding sites for the IDA-Cu lipid-streptavidin interaction.

We have grown streptavidin crystals under both constant-surface-pressure and constant-area conditions. Crystal formation and growth proceeded in a similar fashion in both cases. In both types of experiments, protein binding was followed by changes in the surface pressure-molecular area behavior of the lipid monolayer (see Chapter 3). Typical plots of the changes in surface pressure and molecular area as functions of time are shown in Figures 4.5 and 4.6, respectively. Increases in surface pressure began immediately after injection of protein. The first aggregates appeared after a pressure increase of 1.8 mN/m. As the crystals grew, the surface pressure continued to increase and reached saturation (8 mN/m) at about the same time the crystals stopped growing and the monolayer became stiff. Similar results were obtained in constant-surface-pressure experiments. The first aggregates appeared after an area increase ($\Delta A/A$) of 0.20, the area continued to increase as the crystals grew, and the area change reached saturation ($\Delta A/A = 0.45$) as the streptavidin crystals reached their maximum size.

Injection of the soluble metal chelator ethylenediaminetetraacetate (EDTA) into the subphase beneath mature streptavidin crystals caused the protein crystals to disappear within 15-30 minutes (Figure 4.3.f). EDTA is a strong chelator for divalent metal ions, binding Cu^{2+} very tightly, with $\log K = 18.7$ compared to $\log K = 10.6$ for IDA [31]. In addition, no crystals were observed, and, in fact, no protein bound to the interface after injection of the same amount of streptavidin underneath a DOIDA monolayer in the absence of Cu^{2+} . These results indicate that IDA-lipid binding of streptavidin requires the presence of copper ions.

Streptavidin crystallization on PSIDA-Cu monolayers.

We have also utilized the fluorescent lipid PSIDA-Cu to promote 2D streptavidin crystallization. Adsorption and crystallization on Langmuir monolayers of PSIDA-Cu were similar to those on DOIDA-Cu monolayers. A sequence of Brewster angle micrographs illustrating the process on a PSIDA-Cu monolayer at 3 mN/m is given in Figure 4.7. As on DOIDA-Cu, the crystals were rectangular and maintained this characteristic shape throughout their growth. Ultimately, the crystals covered most of the interface, the monolayer became stiff and the crystal growth stopped. This process was again observed by fluorescence microscopy of rhodamine-labeled streptavidin, as shown in Figure 4.8. In this experiment, a high density of very small, highly aggregated tiles was initially observed. The individual domains within the aggregates grew in size over time, largely maintaining the characteristic square corners. Some very small, isolated tiles were observed, but these were rare (Figure 4.8.c). This different crystallization occurred occasionally with both lipids and, therefore, was not caused by the different lipid tails. The cause of this alternative crystallization process is not fully understood at this time, but is thought to be a result of contaminants (introduced by the protein solution, the lipid solution or the subphase buffer) which limit the crystal growth. As with DOIDA-Cu-grown crystals, addition of EDTA (50 μ M) to the subphase resulted in complete disappearance of streptavidin from the interface within 30 minutes.

Because of the pyrene moiety in the PSIDA tails, the monolayer and protein can be observed simultaneously. In the absence of protein, the pyrene fluorescence showed only a homogeneous bright field. Due to the very low fluorescence intensity of PSIDA-Cu monolayers at 3 mN/m, no emission filter was used; both monomer and excimer emissions are collected and reach the camera system. The image intensifier is insensitive to UV wavelengths, however, and the images represent primarily pyrene excimer emission. When streptavidin crystals were present, the shapes of the crystals could be visualized in the pyrene fluorescence as well as by rhodamine fluorescence. In fact, a contrast inversion

was observed when switching between rhodamine and pyrene fluorescence images (Figure 4.9). The monolayer regions outside the protein crystals (dark in rhodamine images) showed the typical bright pyrene emission intensity, while the lipid fluorescence is greatly reduced in regions containing the bright streptavidin crystals. As pyrene fluorescence was well known to be highly dependent on its local environment [32, 33], the changes in pyrene emission indicate changes in the membrane properties induced by the presence of the protein crystals. The decrease in pyrene excimer intensity could be the result of a decrease in the lateral lipid mobility caused by steric interactions of the lipid headgroups with the underlying protein. Alternatively, lipids may be expelled from the crystalline regions due to penetration of the protein (*vide infra*), or the pyrene fluorescence may be quenched by the presence of the protein.

Visualization of streptavidin crystals by polarized fluorescence microscopy.

Polarized fluorescence microscopy allows investigation of the crystallinity and visualization of the relative orientations of the streptavidin aggregates. In this technique, polarizing filters are placed in the excitation and emission light paths with their directions of polarization perpendicular to one another (i.e., crossed polarizers). Fluorophores in which the absorption dipole is aligned with the polarization of the excitation light are selectively excited and are therefore observed. Fluorophores in which the absorption dipole is aligned perpendicular to the excitation light do not absorb and therefore do not emit fluorescence. Those with an intermediate orientation will emit fluorescence with an intensity between the two extremes. An amorphous phase, in which fluorophores are randomly aligned, will emit fluorescence of equal brightness regardless of the orientation of the polarizing filters. In contrast, a crystalline phase is expected to display a single orientation of fluorophores. Thus, a crystal which appears bright when viewed through a particular orientation of crossed polarizers will appear dark when both polarizers are rotated by 90°.

Pairs of fluorescence micrographs showing DOIDA-Cu-bound streptavidin tiles viewed under perpendicular orientations of crossed polarizers are shown in Figure 4.10. These tiles, while appearing largely aggregated with one another, retained the characteristic sharp edges and square corners of crystals grown on IDA-Cu lipid monolayers. The inverted contrast in each pair of images is readily apparent, suggesting the domains are indeed crystalline. Surprisingly, the crystals appear to be oriented with one another within individual aggregates, indicating the crystals either arose from a single nucleation site or became aligned as they aggregated (see Discussion).

Effect of biotin on streptavidin binding and crystallization.

Mature streptavidin crystals, grown on both DOIDA-Cu and PSIDA-Cu monolayers, rapidly disappeared upon injection of biotin into the subphase at 4- to 40-fold molar excess over the total protein concentration. Even when stoichiometric quantities of biotin (4-fold excess) were added, the protein disappeared from the interface within five minutes. In addition, when streptavidin was pre-loaded with biotin prior to injection beneath the DOIDA-Cu monolayer, no binding or crystallization was observed. These results are rather surprising as biotin is not expected to effect the IDA-Cu-His complex directly, and its binding by streptavidin causes only small perturbations in the protein structure [23] (*vide infra*).

A variety of biotinylated molecules, spanning a wide range of molecular sizes, was also capable of disrupting the crystals. Addition of a biotinylated fluorescein molecule (see Experimental), at a 4-fold molar excess over the streptavidin concentration, resulted in disappearance of protein from the interface within 5 minutes. Injection of biotinylated lysozyme to 16-fold molar excess (30 nM streptavidin, 500 nM lysozyme) caused the disappearance of streptavidin from the interface within 15 minutes. Native, un-biotinylated lysozyme in the same concentration also disrupted streptavidin crystals. Without biotin, however, the process of crystal disruption required greater than 45 minutes and was

accompanied by a large increase in the surface pressure to > 20 mN/m. Thus, streptavidin crystal disruption by native lysozyme appears to occur by a different mechanism. Most likely, lysozyme binds the IDA-Cu headgroups outside the streptavidin crystals, increasing the surface pressure; lysozyme is known to have significant affinity for immobilized metal [34]. The surface pressure increase could be responsible for the crystal disruption. Finally, a bis-biotin linker was prepared and loaded into a solution of streptavidin. Thus, each of the biotin binding pockets contained one bis-biotin molecule, and the resulting protein displayed four biotin moieties capable of further binding additional streptavidin. Addition of a 4-fold molar excess (with respect to apo-streptavidin initially present) of bis-biotin streptavidin beneath mature crystals of apo-streptavidin induced disruption of the crystals and complete disappearance of protein from the interface in approximately 45 minutes.

From the fluorescence micrographs and BAM, it is unclear whether biotin is interfering with streptavidin's ability to coordinate the immobilized metal ions or is inhibiting streptavidin crystallization. Thus, the ability of streptavidin, with and without biotin, to coordinate IDA-Cu was investigated by analytical IMAC. Apo-streptavidin was strongly retained on an IDA-Cu-derivatized support; 40 mM imidazole was required for elution (Figure 4.11). In contrast, when each of the four binding pockets was loaded with biotin, the protein was only poorly retained, eluting from the column at the start of the imidazole gradient. When streptavidin was loaded with only one biotin per protein tetramer, heterogeneous binding to the IDA-Cu support was observed with a large fraction of the protein eluting as the apo-protein (46%) at 40 mM imidazole, a second large fraction (40%) eluting at the start of the gradient and a smaller fraction (14%) eluting at an intermediate imidazole concentration of 24 mM. Thus, it appears binding of biotin interferes with the ability of streptavidin to coordinate metal ions.

Discussion

Streptavidin has been shown to form aggregated protein domains on Langmuir monolayers of the metal-chelating lipids DOIDA-Cu and PSIDA-Cu *via* coordination of the metal ions by surface histidine side chains. Several lines of evidence point to the crystallinity of the streptavidin domains. Their sharp edges and consistent shape, both during growth in a single experiment and from experiment to experiment, strongly suggest the domains are crystalline. Secondly, the polarized fluorescence microscopy results (Figure 4.10) support the ordering within the streptavidin domains. Finally, Fourier analysis of an electron micrograph of DOIDA-Cu-bound streptavidin aggregates indicates the protein is ordered and has shown lattice parameters identical to those of biotin lipid-bound streptavidin crystals [35]. In addition, a filtered image in negative stain produced a low resolution projection map of the electron density [35] very similar to the known 2D projection of biotin-bound streptavidin crystals at a comparable resolution [21, 36].

Protein concentrations in the subphase as low as 20 nM were sufficient to produce crystals. Such a low concentration is surprising given that only two simultaneous IDA-Cu-histidine interactions are possible (*vide infra*). A single IDA-Cu-histidine interaction provides a $K_d = 10^{-3.5}$ M [18, 37]. Therefore, in the absence of any entropic losses, the *maximum* affinity for two simultaneous interactions would be $K_d = 10^{-7.0}$ M. Protein concentrations significantly less than K_d are sufficient for crystal growth. This could indicate that protein-protein interactions play a significant role in stabilization of streptavidin binding to the IDA-Cu lipid monolayer. In addition, at pH 7.8, other basic groups on the protein, such as lysine and the N-terminus, can participate in metal coordination and may contribute to the affinity of streptavidin for the IDA-Cu lipid surface.

Crystallization on IDA-Cu lipids was performed at surface pressures near 3 mN/m. Higher pressures greatly reduced the formation of crystals. In fact, compression of the monolayer to pressures of 15-20 mN/m was sufficient to destroy the mature crystals. Such low surface pressures in the IDA-Cu lipid monolayers may be required in order to provide

sufficient mobility of the lipid and bound protein. However, this seems unlikely, as DOIDA-Cu monolayers appear to be in the liquid state at all pressures and are thus expected to be very mobile. Streptavidin crystallization on biotinylated monolayers is known to depend on the length of the spacer attaching the biotin to the lipid backbone. Very long spacers allow crystallization over a large range of surface pressures, shorter spacers allow crystallization only in the fluid state, and, with no spacer, crystallization occurred only in non-compressed monolayers [21]. This was believed to be due to increased penetration of the protein into the monolayer as the length of the spacer was decreased. Due to differences in (1) the IDA-Cu and biotin lipid structures and (2) the binding site on the protein, it is unclear in which class of spacer length the IDA-Cu lipids should be placed. Nevertheless, the inhibition of crystallization with increasing surface pressure is likely a result of decreasing ability of streptavidin to penetrate the IDA-Cu monolayer. A requirement for partial penetration of the protein into the lipid bilayer also explains the increase in surface pressure or monolayer area observed during crystallization (Figures 4.5 and 4.6). We might expect crystallization on IDA-Cu monolayers to be more sensitive to surface pressure than that on biotin lipid monolayers due to the vastly larger affinity of streptavidin for the biotin lipids. This larger binding energy will allow the protein to remain attached to the lipids at higher surface pressures where steric interactions with the monolayer make adsorption less favorable. In fact, streptavidin crystals grown on biotin lipid may be compressed to surface pressures as high as 32 mN/m with no effect on the crystal morphology [29].

Crystals grown on both DOIDA-Cu or PSIDA-Cu monolayers tended to aggregate with one another. In general, the contacts between neighboring crystals occurred between two corners or two flat sides. Contact between a corner and a flat side was rare. This seems to indicate that not only are the crystals aggregating but they appear to be aligning with one another. This supposition is supported by the images of crystal aggregates viewed through crossed polarizers (Figure 4.10). Within each irregularly shaped

aggregate, many of the neighboring crystals show the same brightness and contrast inversion upon rotation of the polarizers, indicating they are similarly aligned. One further observation is that, early in the crystallization process, the aggregates appear to be made up from a large number of very small, rectangular crystals. Near the end of the crystallization process, a smaller number of large crystals is observed. This seems to indicate that the individual crystals in the aggregates “fuse” with their neighbors, ultimately becoming a single crystalline domain.

2-D crystal morphology: tiles versus “H’s.”

In contrast to the rectangular-shaped streptavidin crystals grown on IDA-Cu lipid monolayers, those grown on biotin lipid monolayers display a characteristic H-shape [38, 39]. The different crystal morphology could be a result of different intermolecular contacts or a different orientation of the protein with respect to the monolayer. This would be consistent with the ability of streptavidin to crystallize in several polymorphs in three dimensions [23, 40]. However, through the use of the H87A and H127C mutants, we have determined that His 87 is responsible for binding of streptavidin to IDA-Cu. The histidine at position 87 lies very near the biotin binding pocket (Figure 4.1). The positioning of two His 87 residues on the same face of the protein will result in approach of the IDA-Cu lipid headgroups from a similar direction as biotin lipids bound in the binding pockets. Therefore, we expect streptavidin bound to IDA-Cu- and biotin-lipid monolayers will share the same orientation with respect to the monolayer. A similar orientation of the individual proteins in the two types of crystals implies similar intermolecular contacts, and therefore different polymorphs are unlikely. In addition, the electron diffraction results of Chang and Kornberg (private communication) indicate identical packing of IDA-Cu-bound and biotin-bound streptavidin crystals, and the electron density map further supports the identical orientation and inter-protein contacts in these two cases.

Using fluorescence and electron microscopies, Ku *et al.* have made a detailed analysis of the intermolecular interactions in biotin lipid-grown streptavidin crystals and their relationship to the overall crystal morphology [36, 41]. In biotin lipid-grown crystals, the biotin binding pockets closest to the monolayer are presumably filled with biotin while those facing the solution are empty. This results in two different types of protein-protein contacts: those in which the contacting monomers are free of biotin and those in which the contacting monomers are loaded with biotin (Figure 4.12, A). Biotin binding is known to result in subtle changes in the streptavidin crystal structure [23], and seemingly the structural changes alter the interactions between neighboring proteins in the 2D crystal. The unfilled-unfilled monomer contacts are preferred, and this asymmetry produces a preferred growth direction resulting in the typical H-shape.

Crystals of apo-streptavidin grown on IDA-Cu lipid monolayers contain no biotin; only unfilled-unfilled monomer contacts exist in this system. Thus, there is no asymmetry in the molecular interactions, and there should be no preferred growth direction. In fact, the IDA-Cu-bound crystals are symmetric (rectangular), reflecting the assumed square molecular lattice as would be expected. Given this proposed explanation for 2D streptavidin crystal morphology, it may be possible to control crystal shape by selective loading of the protein with biotin; i.e., by preparing streptavidin loaded with only two equivalents of biotin, both of which are located on the same side of the tetramer. Such a protein preparation may be possible by employing chimeric streptavidin tetramers as described by Chilkoti *et al.* [30]. When 2D crystals of this streptavidin variant are grown on biotin lipid monolayers, all four binding pockets would be expected to be filled. Thus, only one type of protein-protein contact would exist, and rectangular crystals would be produced (Figure 4.12, B). In contrast, on IDA-Cu monolayers, both filled-filled and unfilled-unfilled monomer contacts would be expected, potentially resulting in H-shaped crystals (Figure 4.12, D). Note that this result will depend upon the ability of streptavidin to coordinate IDA-Cu with two binding sites filled (*vide infra*).

Biotin-induced structural changes affect metal affinity of streptavidin.

Addition of biotin to the subphase below mature streptavidin crystals induced rapid disappearance of the protein from the monolayer surface. In addition, streptavidin pre-loaded with biotin failed to form crystals. Presumably, these effects are due to binding of biotin in the streptavidin binding pockets. Initially, it was not clear whether biotin binding affected the affinity of streptavidin for the IDA-Cu lipid or whether it affected the intermolecular contacts and, therefore, the protein's ability to crystallize. That the ability to crystallize was affected seems unlikely as streptavidin is known to crystallize on biotin-lipid monolayers, and the two empty sites of these crystals can be loaded with biotin or biotin-derivatives in limited quantities without affecting the crystals. However, exposure of biotin lipid-grown crystals to higher concentrations of free biotin (200 nM) resulted in the rapid disruption of the crystals, most likely due to biotin competing with the weaker binding biotin lipid [21].

Immobilized metal ion affinity chromatography showed that biotin binding essentially eliminated the affinity of streptavidin for the immobilized copper (Figure 4.11). How the biotin interferes with metal binding is unclear, however. While the biotin binding pocket and His 87 are on the same face of the protein, biotin is buried deeply in its pocket; direct interaction between bound biotin and His 87 is unlikely. For the same reason, steric interference between the lipid (or chromatography support) and biotin is also unlikely. Weber *et al.* have solved the three-dimensional crystal structure of streptavidin in the presence and absence of biotin [23, 24] and found several significant structural changes upon biotin binding. Small changes in quaternary structure were observed, including a slight rotation of the subunits relative to one another. In addition, two surface loops, residues 45-50 and 63-69, were resolved in the biotin-bound structure, yet were disordered and therefore not located in the absence of biotin. Loop 63-69 on the "bottom" of a particular subunit lies near His 87 on the "top" of the neighboring subunit (where "top" corresponds to the side on which the entrance to the biotin binding pocket is located).

Locking this surface loop into place by binding of biotin may reduce the accessibility of His 87. Another factor which should be considered is that biotin binding may interfere with any conformational changes His 87 must undergo in order to bind IDA-Cu. For instance, a neighboring residue, Ser 88, strongly interacts with one of the carboxyl oxygens on the tail of biotin which may restrict the movement of His 87.

The number of biotin binding sites which must be occupied in order to disrupt the crystals is unknown. In addition, it is interesting to know whether binding to the two exposed pockets below the crystal is sufficient to disrupt the crystals, or whether biotin must reach the binding sites closest to the lipid monolayer. The ability to decorate the crystal surface by binding additional biotinylated molecules to the exposed pockets would be highly desirable as this can lead to a variety of applications [39, 42, 43]. Biotin in the aqueous subphase obviously has free access to the two binding pockets facing the solution. It should be understood that biotin has access to the binding pockets between the protein crystal and the monolayer, as well. From the two-dimensional streptavidin crystal structure [11, 21], it is known that the crystal contains a large amount of water in the $\sim 50 \times 40 \text{ \AA}^2$ "pores" between the proteins (c.f. Figure 4.12) [11], and it should be relatively easy for biotin to reach the "top" of the crystal. This is evidenced by the disruption of biotin-bound crystals in the presence of large biotin concentration in the subphase [21]. In addition, biotin has been shown to disrupt streptavidin crystals grown on lipid monolayers containing the weaker binding desthiobiotin ($K_a = 10^{13} \text{ M}^{-1}$) [44]. This accessibility of the "top" of the crystal to small molecules in the subphase also explains the uniform dissolution of crystals in the presence of EDTA; e.g., small molecules have access to the middle of the crystal as well as the edges, simultaneously. There will likely be some hindrance to diffusion to the binding pocket, however, due to the close proximity of the protein and the lipid monolayer. Nevertheless, the slightly larger fluorescein biotin compound, which might be expected to have more difficulty reaching the "top" binding pockets, disrupts

DOIDA-Cu-bound streptavidin crystals as quickly as biotin. This may indicate biotin binding to the freely accessible “bottom” pockets plays a role in disruption of the crystals.

In order to confirm this supposition, the ability of biotinylated macromolecules to disrupt streptavidin crystals was investigated. Lysozyme is a relatively small protein (MW 14,300) of dimensions $45 \times 30 \times 30 \text{ \AA}^3$. This protein, therefore, is small enough to fit into the pores between the streptavidin molecules in the crystal, but would likely not have access to the “top” binding pockets in the intact, mature crystals. Given $\sim 3600 \text{ \AA}^2$ per streptavidin protein in the crystal, two lysozyme molecules are expected to be able to bind both biotin binding pockets simultaneously. A biotinylated lysozyme preparation was capable of disrupting the mature streptavidin crystals in a matter of 15 minutes. Similarly, the bis-biotin-containing streptavidin disrupted mature apo-streptavidin crystals within 45 minutes of injection into the subphase. Streptavidin is too large to fit into the crystalline pores. The geometrical identity between the bis-biotin streptavidin and apo-streptavidin, however, allows 1:1 binding, filling both of the exposed biotin-binding pockets of the DOIDA-Cu-bound protein.

As mentioned above, access of the protein-bound biotin to the “top” biotin binding pockets in the intact crystal is hindered by the geometry of the crystal and the monolayer. In order for these biotinylated macromolecules to bind to the crystalline streptavidin at the “top” binding pockets, it would be necessary for the streptavidin to unbind from the monolayer and the crystal, exposing the “top” of the protein. This scenario seems unlikely given the apparently strong binding of streptavidin within the crystals; i.e., 20 nM protein in the subphase is sufficient to promote crystallization, indicating a rather high affinity of streptavidin for the IDA-Cu monolayers. However, both of these biotinylated proteins have free access to the “bottom” biotin binding pockets of the crystal, which are exposed to the aqueous subphase. This suggests that biotin-induced structural changes are transduced across the protein such that binding of biotin on one side of the streptavidin molecule inhibits metal coordination by His 87 on the opposite side of the protein. This is most

likely due to the slight biotin-induced changes in quaternary structure known from the 3D crystal structure [23]. Alternatively, binding of biotin may disrupt the IDA-Cu-grown crystals through disturbance of the protein-protein contacts. As described above, binding of biotin to two binding pockets on biotin-lipid monolayers negatively affects the protein-protein contacts sufficiently to result in an asymmetry in the crystal growth directions. Due to the weaker affinity of streptavidin for the IDA-Cu lipids, binding of the protein to the monolayer and the stability of the crystal depend more strongly on the inter-protein contacts. Disturbance of even two (of the four) protein-protein contacts may be sufficient to disrupt the crystals and weaken the apparent affinity of the protein for the IDA-Cu lipid monolayer.

Conclusion

Metal ion coordination by surface-accessible histidine side chains, either genetically engineered or naturally occurring, can provide a general method for targeting of proteins to metal-chelating lipid membranes, and the ability to immobilize protein on a mobile lipid monolayer is the first step toward 2D protein crystallization. In fact, in the present study, coordination of two histidines to IDA-Cu lipid monolayers leads to 2D crystallization of streptavidin. This represents the first time a protein has been crystallized based on metal coordination of naturally occurring histidines. Two-dimensional protein crystallization has, to date, been hindered by the need for a unique affinity lipid for each protein studied. In contrast, surface-accessible histidine residues are relatively common, and this approach will allow 2D crystallization studies of a wide variety of proteins. In addition, with genetic engineering, histidine residues can be created on proteins at will, potentially extending this approach to virtually any protein of interest. Thus, metal-chelating lipids have the potential to greatly accelerate the investigation of proteins for 2D crystallization and should facilitate the exploration of organized protein arrays as functional materials.

Experimental

Materials.

The metal chelating lipids DOIDA [25, 45] and PSIDA [15] were synthesized as described previously (see Chapter 2). Fluorescein biotin (5-((N-(5-(N-(6-(biotinoyl)-amino)hexanoyl)amino)pentyl)thioureydyl)fluorescein), was obtained from Molecular Probes, Inc. (Eugene, OR). Native streptavidin was obtained from Boehringer-Mannheim as a lyophilized powder; lot numbers were 13381668-33, 13381687-33, and 13556252-35. Streptavidin mutants were prepared by Dr. Ashutosh Chilkoti and Dr. Patrick Stayton, University of Washington-Seattle, as described [25, 30]. Chloroform, methanol, ethanol (all A.C.S. HPLC grade), hexane (99+%) and CuCl_2 (99.999%) were obtained from Aldrich (Milwaukee, WI). 3-(N-morpholino)propanesulfonic acid (MOPS) was obtained from Sigma (St. Louis, MO).

Streptavidin crystallization.

Crystallization experiments were performed under several different conditions with equal success. Experiments in which crystallization was observed by Brewster angle microscopy were performed in the laboratory of Dr. Viola Vogel, University of Washington-Seattle, and have been described elsewhere [25, 28]. Experiments in which crystallization was observed by fluorescence microscopy were performed in our laboratory under two sets of conditions; constant pressure and constant area (results were equivalent). However, some procedures were consistent between labs. Lipid solutions were prepared in chloroform. The IDA lipids were pre-metalated by adding an aliquot of concentrated CuCl_2 in methanol to the lipid solutions. Cu^{2+} excess in the lipid solution is $\leq 10\%$. The monolayer subphase was buffered with 20 mM MOPS, 250 mM NaCl, pH 7.8. The buffer was shaken with chloroform to remove organic impurities and, subsequently, shaken with hexane to remove traces of chloroform. Streptavidin concentrations were

determined by UV-vis absorption spectroscopy at 280 nm using an extinction coefficient of $136,000 \text{ M}^{-1} \text{ cm}^{-1}$.

Constant-pressure experiments were performed in a computer-controlled Langmuir trough (KSV Instruments, Helsinki) with a surface area of $75 \times 230 \text{ mm}^2$. The entire film balance was placed inside a Plexiglas box, and the atmosphere was purged with Ar. The surface pressure was measured by the Wilhelmy plate method using plates cut from filter paper (Whatman, No. 1). The plate was rinsed thoroughly with chloroform and calibrated with stearic acid prior to use. Monolayers were compressed at a constant rate of $3 \text{ \AA}^2/\text{molecule} \cdot \text{min}$. A 15-minute waiting period prior to compression allowed for solvent evaporation. Once the desired surface pressure (3 mN/m) was attained, the instrument was switched to constant-pressure mode, and the monolayer was allowed to equilibrate for 15-30 minutes. The desired amount of protein, dissolved in the above buffer, was injected into the subphase either behind the barrier or through the monolayer and allowed to mix by diffusion. Changes in surface area were recorded as a function of time. After each experiment, the trough was thoroughly cleaned with detergent (RBS 35), Milli-Q purified water, and chloroform. All measurements were performed at room temperature, $20 - 27^\circ \text{C}$.

Constant-area experiments were performed in a Petri dish hydrophobized with octadecyltrichlorosilane. Holes cut in the lid of the Petri dish allowed access of the Wilhelmy plate and entrance of Ar. Surface pressure was again measured using plates cut from filter paper. Monolayers were spread dropwise until the desired surface pressure was attained. Time was allowed between drops for stabilization of the surface pressure. Thirty to 45 minutes after spreading, a concentrated streptavidin solution was injected into the 15-mL subphase through the monolayer. Surface pressure was measured as a function of time.

Labeling of proteins.

Streptavidin was labeled with rhodamine succinimidyl ester (RSE) following the standard procedure [46] with a slight modification as described in Chapter 3. Briefly, 10 mM RSE in dry DMF was added dropwise, with stirring, to a solution of 20-100 μ M streptavidin (in 0.1 M NaHCO_3 , 0.1 M NaCl, pH 8.3) to yield a dye/protein ratio of 2:1. The reaction mixture was incubated at room temperature for one hour. Labeled protein was separated from unreacted dye by gel filtration chromatography on pre-packed Sephadex G-25 columns (PD-10, Pharmacia, Uppsala, Sweden) pre-equilibrated with 20 mM MOPS, 250 mM NaCl, pH 7.8.

Lysozyme was simultaneously labeled with rhodamine and tagged with biotin. The procedure was essentially the same as that described above for labeling of streptavidin with rhodamine succinimidyl ester. However, in addition to the RSE, a biotinoyl succinimidyl ester derivative, dissolved in dry DMF, was added to the reaction mixture at a biotin/lysozyme ratio of 8:1. The ability of the biotin-tagged lysozyme to bind streptavidin was confirmed qualitatively. An aliquot of iminobiotin-derivatized agarose was incubated with fluorescein-labeled streptavidin and the gel was separated by centrifugation. The yellow color of the fluorescein was localized in the gel indicating the protein had been bound by the iminobiotin moieties. The supernatant was drawn off, and a solution of rhodamine/biotin-labeled lysozyme was added to the gel. The protein and gel were mixed briefly, and the gel was pelleted by centrifugation. Again, most of the red color of the rhodamine was localized in the gel, indicating the lysozyme was bound to the free biotin pockets of the previously bound streptavidin.

Fluorescence microscopy.

Fluorescence microscopy was performed essentially as described in Chapters 2 and 3. For polarized fluorescence studies, plastic polarizing sheets were placed in the excitation path just before the excitation filter and in the emission path following the emission filters.

The excitation polarizer was rotated manually, while two emission polarizers, oriented perpendicular to one another, were incorporated into a filter slider. Because the monolayer was fluid, and therefore the domains were constantly in motion, the filter orientations were manipulated while recording the fluorescence images on video tape. Individual images of complementary polarizer orientations were later digitized from the tapes.

Immobilized metal affinity chromatography.

The IMAC column (TSK Chelate-G6000XL, 7.5 cm x 7.5 mm, Tosohaas) was regenerated and loaded with Cu^{2+} [47] and subsequently equilibrated with 10 mL of 50 mM sodium phosphate, 0.5 M NaCl, 100 mM imidazole at pH 7.8, followed by 10 mL of the same buffer containing 1 mM imidazole. Experiments were performed at a flow rate of 1 mL/min. After injecting protein (150 μL of 20 μM streptavidin), the column was washed with 6 mL of buffer containing 1 mM imidazole. The protein was then eluted with a linear gradient of imidazole from 1 to 100 mM over 114 mL, and the eluent was monitored by absorbance at 280 nm. The chromatograms have been corrected for the changing baseline due to absorbance of imidazole by subtraction of the trace obtained in the absence of protein.

Protein	Affinity ligand	Type of interaction	Ref.
Anti-DNP IgG	Dinitrophenyl PE ^a	specific	[8]
Cholera toxin subunit B	Ganglioside GM1	specific	[48]
Cholera toxin	Ganglioside GM1	specific	[49]
Ribonucleotide reductase	dATP-amino-caproyl-PE ^a	specific	[50]
Tetanus toxin	Ganglioside GT1	specific	[51]
Annexin VI	DMPE	electrostatic	[52]
Ferritin	PBLH ^b	electrostatic	[53]
RNA polymerase I	Cetyl trimethyl ammonium	electrostatic	[54]
DNA gyrase B	Novobiocin	specific	[55]
F-actin	Stearylamine	electrostatic	[56]
α -Toxin	Platelet membranes	specific	[57]
Streptavidin	Biotin PE ^a	specific	[21]
RNA polymerase II	Stearylamine	electrostatic	[58]
Catalase	PBLH ^b	electrostatic	[59]
Streptavidin	PBLH ^b	electrostatic	[22]
HIV-1 reverse transcriptase	NTA-Ni	metal coordination	[19]
Streptavidin	IDA-Cu	metal coordination	[25]

^aPE, phosphatidyl ethanolamine; ^bPBLH, poly(benzyl-L-histidine).

Table 4.1. Proteins which have been crystallized in two-dimensions on Langmuir monolayers and the identity and type of interaction utilized for targeting the protein to the membrane.

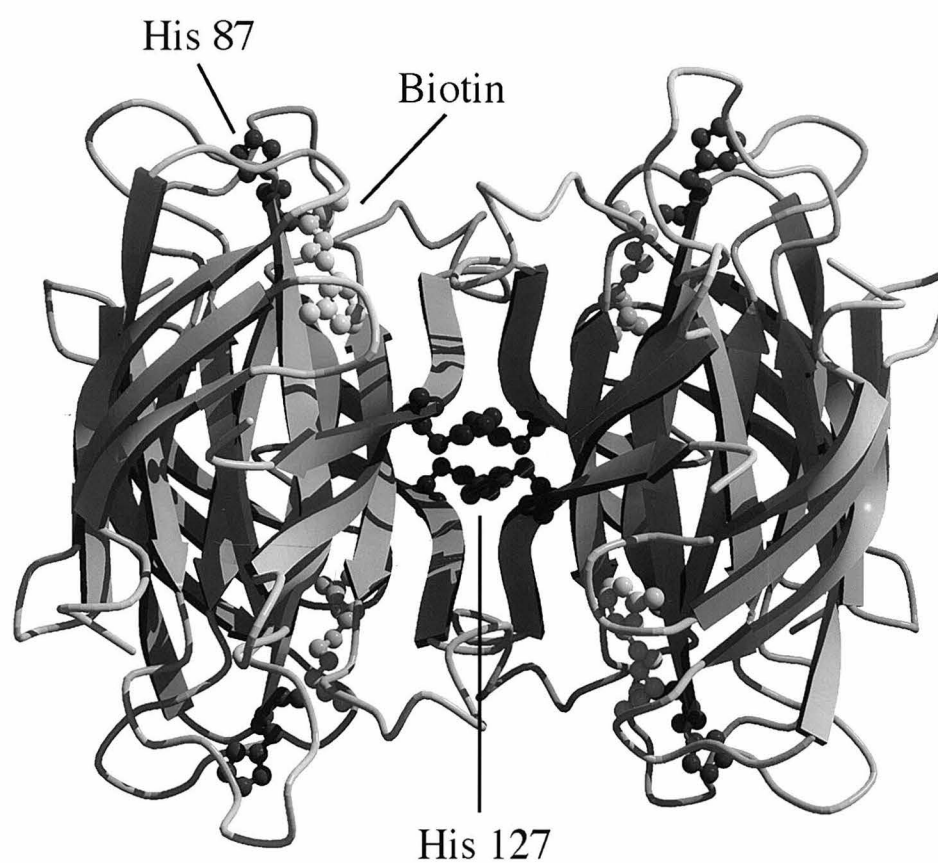


Figure 4.1. Three-dimensional x-ray crystal structure of biotin-bound streptavidin highlighting the relative location of the protein's histidine residues and biotin-binding pockets.

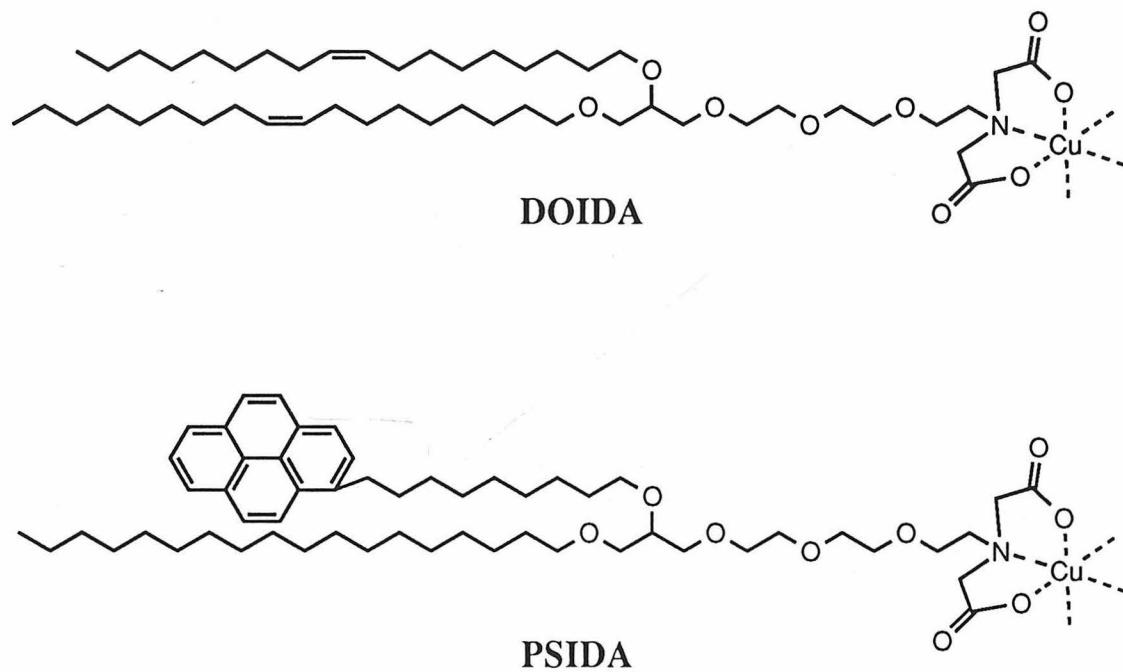


Figure 4.2. Structures of synthetic metal chelating lipids 1,2-dioleyl-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid) (DOIDA) and 1-stearyl-2-(9-(1-pyrene)nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid) (PSIDA) shown as the copper complex.

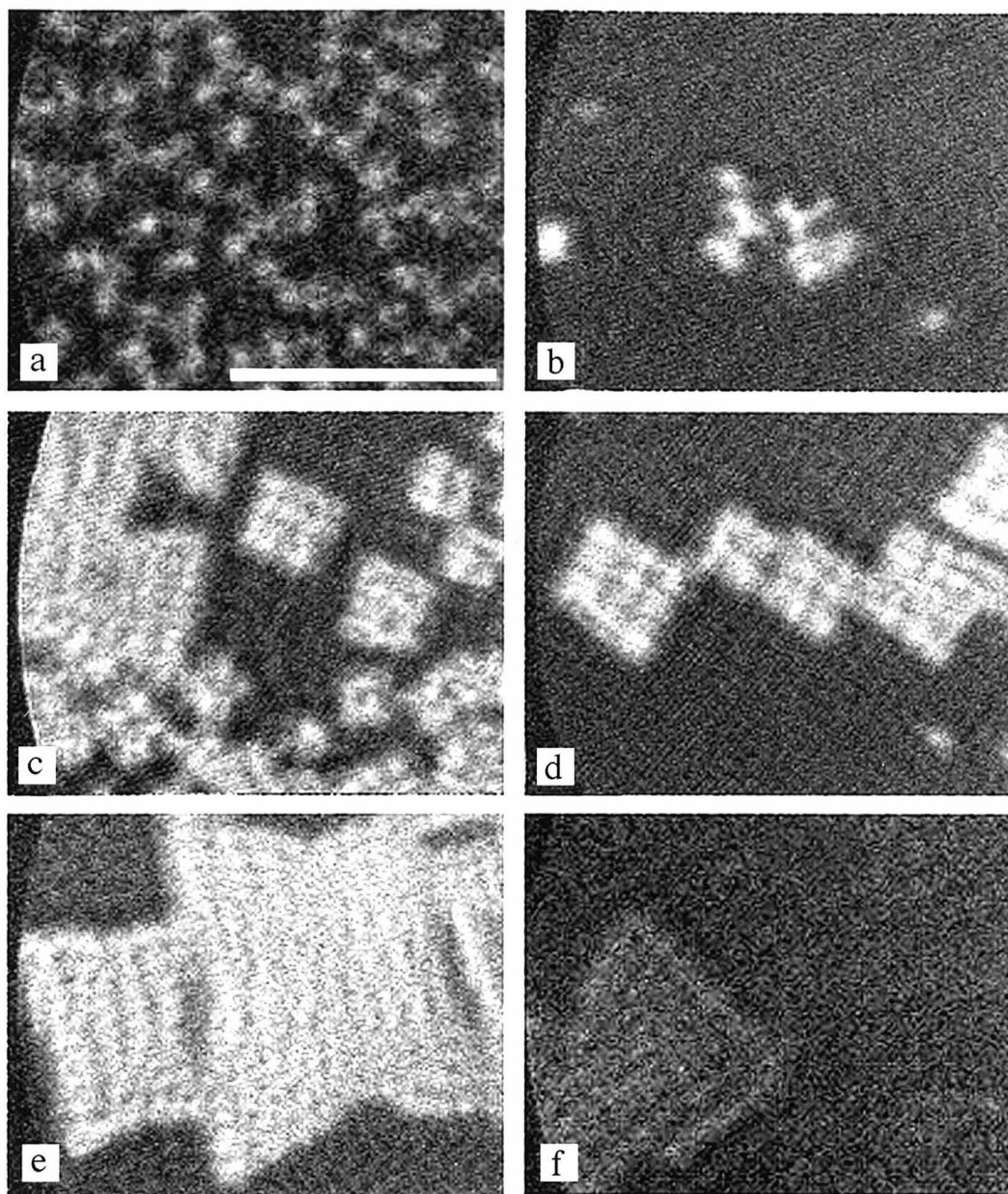


Figure 4.3. Time sequence of BAM images of 2-D streptavidin aggregation on a DOIDA-Cu monolayer (3 mN/m) on buffered subphase (20 mM MOPS, 250 mM NaCl, pH 7.8) at room temperature. Streptavidin concentration is ~ 150 nM. Images were taken 15 (a and b), 35 (c), 90 (d) and 210 (e) minutes after protein injection. Image e was taken following injection of EDTA into the subphase. Scale bar = 100 μm .

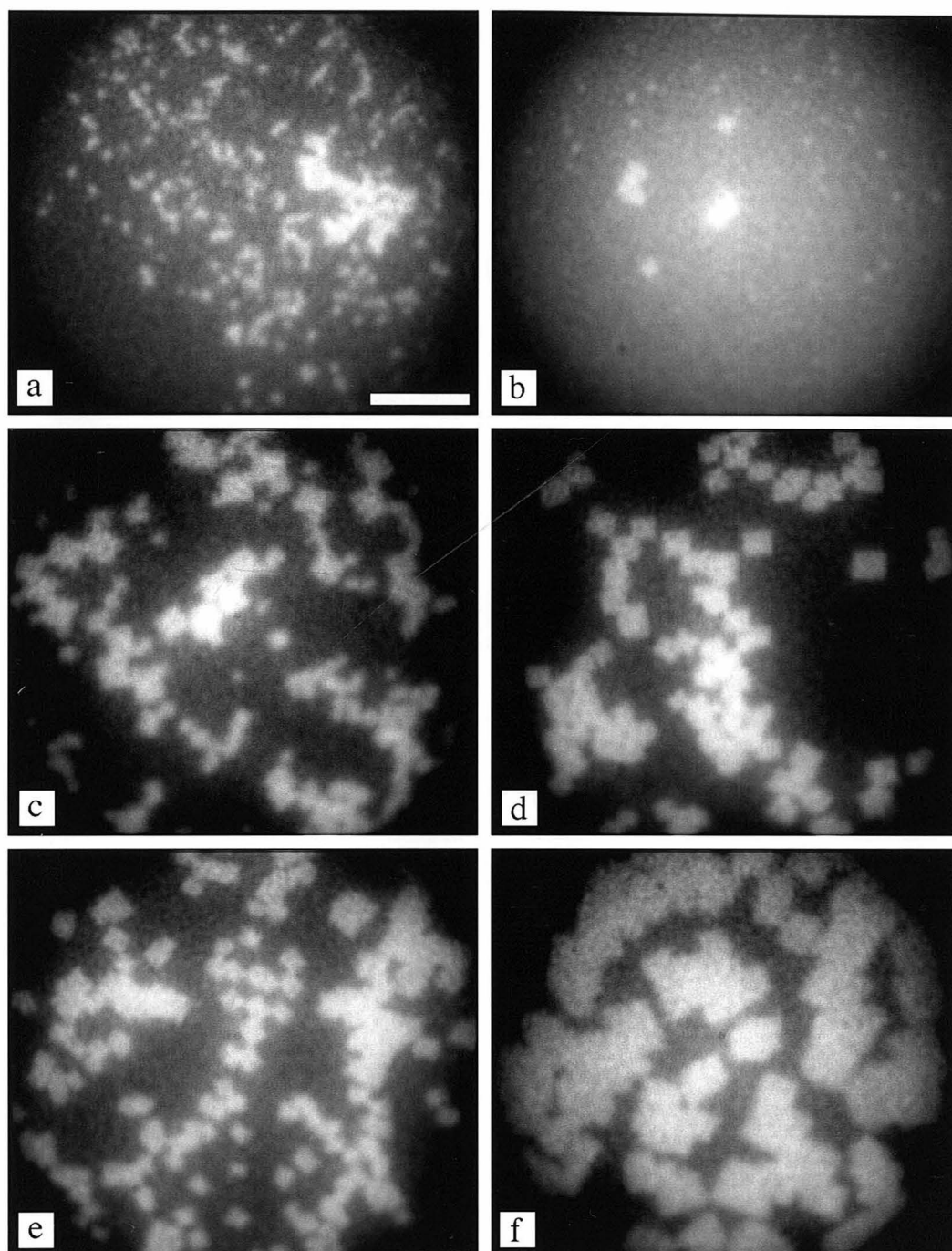


Figure 4.4. Time sequence of fluorescence micrographs of 2-D streptavidin aggregation on a DOIDA-Cu monolayer (3 mN/m) on buffered subphase (20 mM MOPS, 250 mM NaCl, pH 7.8) at room temperature. Streptavidin concentration is ~ 80 nM. Images were taken 115 (a), 125 (b), 150 (c), 190 (d), 200 (e) and 250 (f) minutes after protein injection. Scale bar = $100\ \mu\text{m}$.

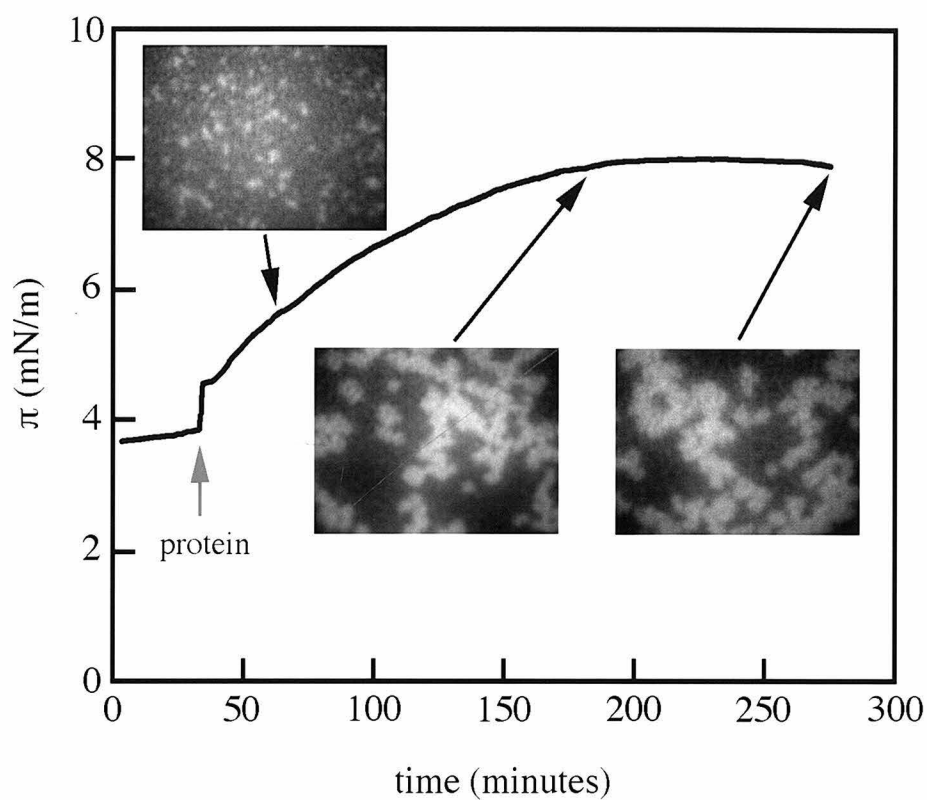


Figure 4.5. Surface pressure of a DOIDA-Cu monolayer on buffered subphase (20 mM MOPS, 250 mM NaCl, pH 7.8) during 2-D streptavidin crystallization. Protein was injected to a total concentration of 19 nM at 34 minutes (gray arrow). Fluorescence micrographs at 62, 184 and 278 minutes show the crystal growth in relation to the pressure change.

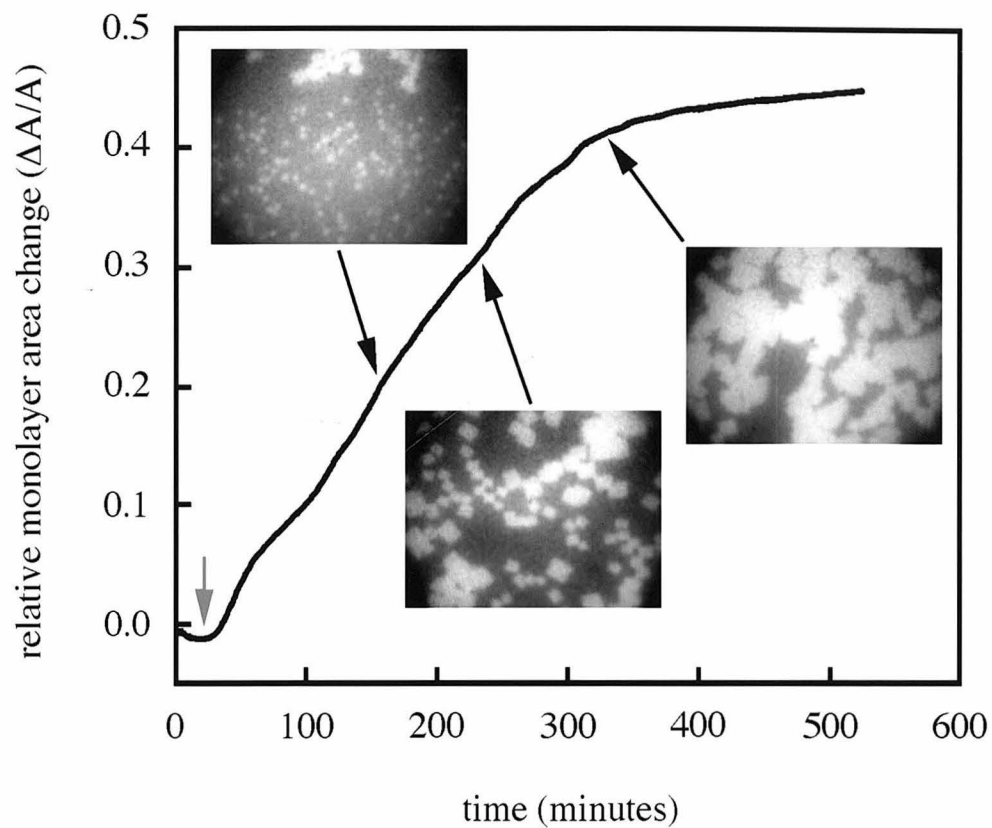


Figure 4.6. Relative monolayer area change of a DOIDA-Cu monolayer on buffered subphase (20 mM MOPS, 250 mM NaCl, pH 7.8) during 2-D streptavidin crystallization. Protein was injected to a total concentration of 80 nM at 22 minutes (gray arrow). Fluorescence micrographs at 150, 220 and 270 minutes show the crystal growth in relation to the area change.

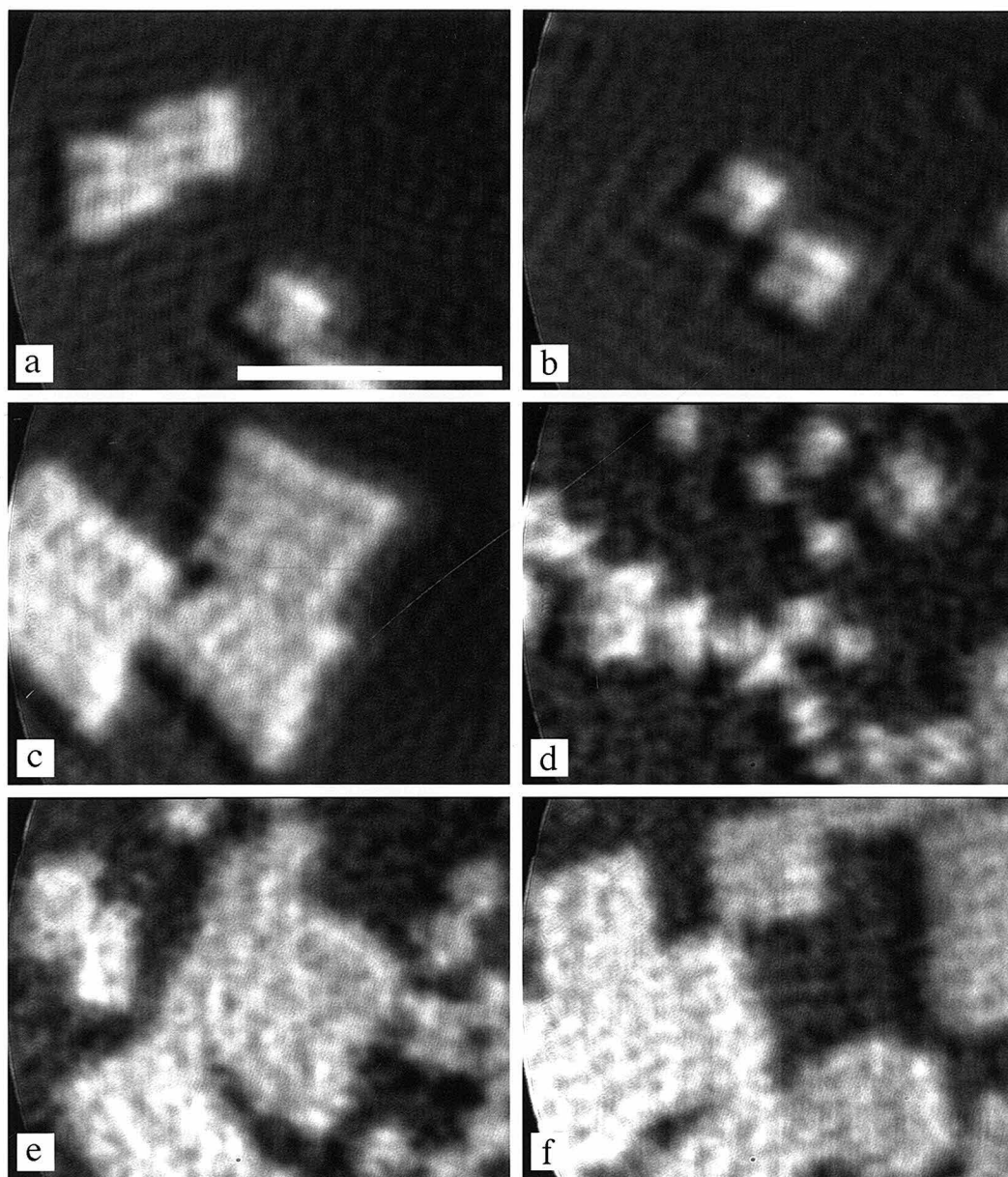


Figure 4.7. Time sequence of BAM images of 2-D streptavidin aggregation on a PSIDA-Cu monolayer (3 mN/m) on buffered subphase (20 mM MOPS, 250 mM NaCl, pH 7.8) at room temperature. Streptavidin concentration is ~ 120 nM. Images were taken 40 (a), 56 (b), 61 (c), 72 (d), 83 (e) and 95 (f) minutes after protein injection. Scale bar = $100\ \mu\text{m}$.

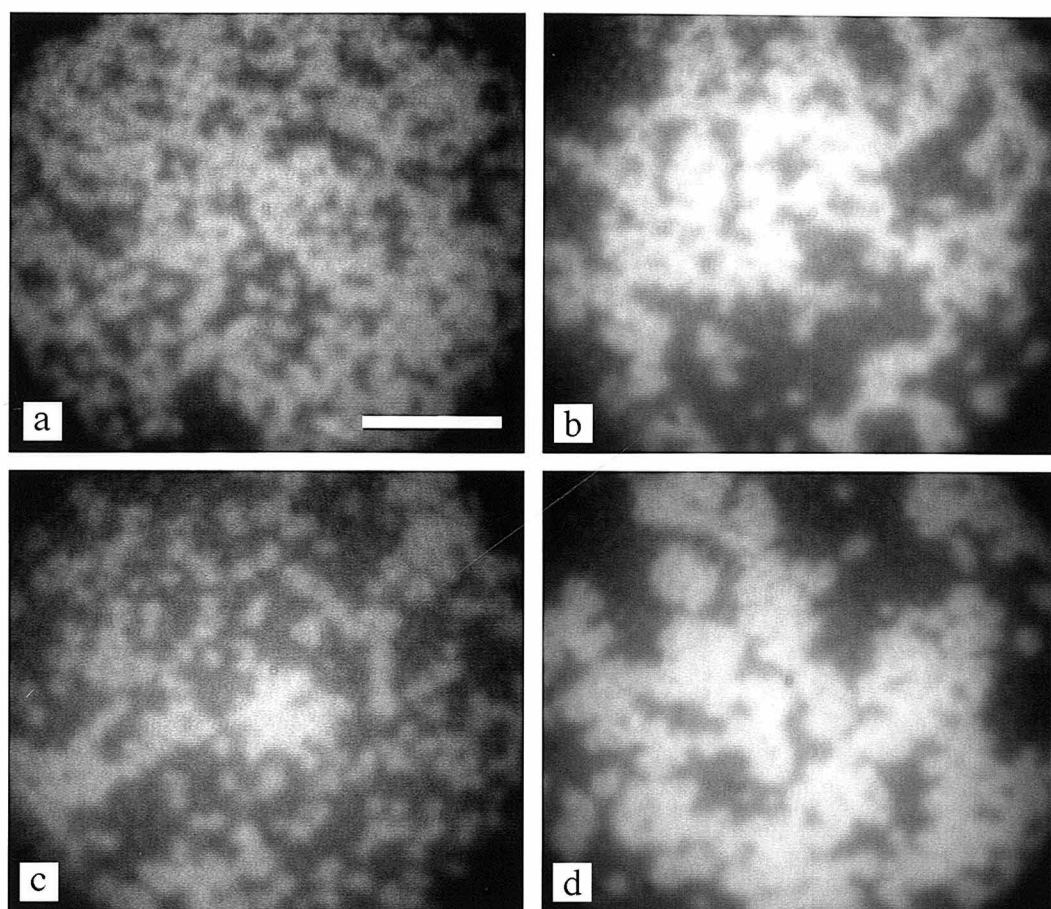


Figure 4.8. Time sequence of fluorescence micrographs of 2-D streptavidin aggregation on a PSIDA-Cu monolayer (3 mN/m) on buffered subphase (20 mM MOPS, 250 mM NaCl, pH 7.8) at room temperature. Streptavidin concentration is ~40 nM. Images were taken 120 (a), 125 (b), 137 (c) and 229 (d) minutes after protein injection. Scale bar = 100 μm .

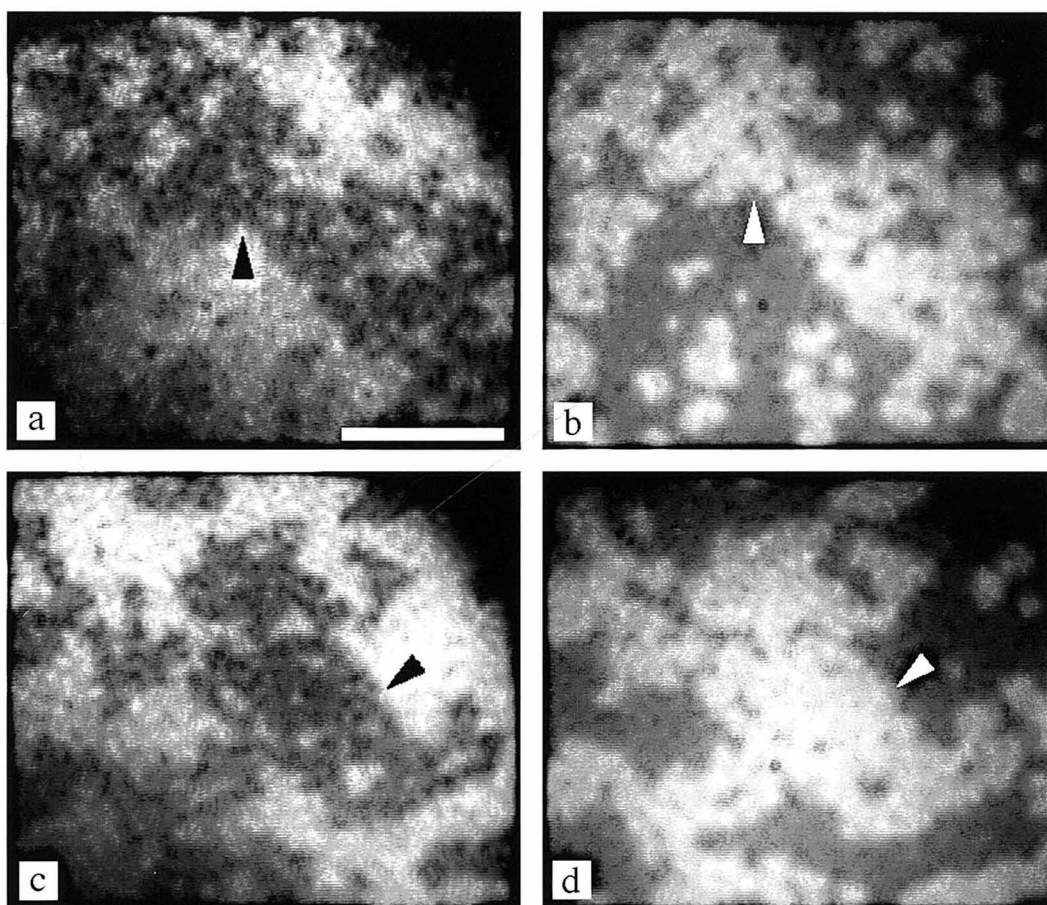


Figure 4.9. Comparison of rhodamine (images a and c) and pyrene (images b and d) fluorescence from PSIDA-Cu monolayer at 3 mN/m on buffered subphase (20 mM MOPS, 250 mM NaCl, pH 7.8) in the presence of streptavidin crystals. Streptavidin concentration is ~30 nM. White arrows indicate equivalent points on the monolayer in each of the pairs of images. The pyrene and rhodamine fluorescence show inverted contrast. Scale bar = 100 μm .

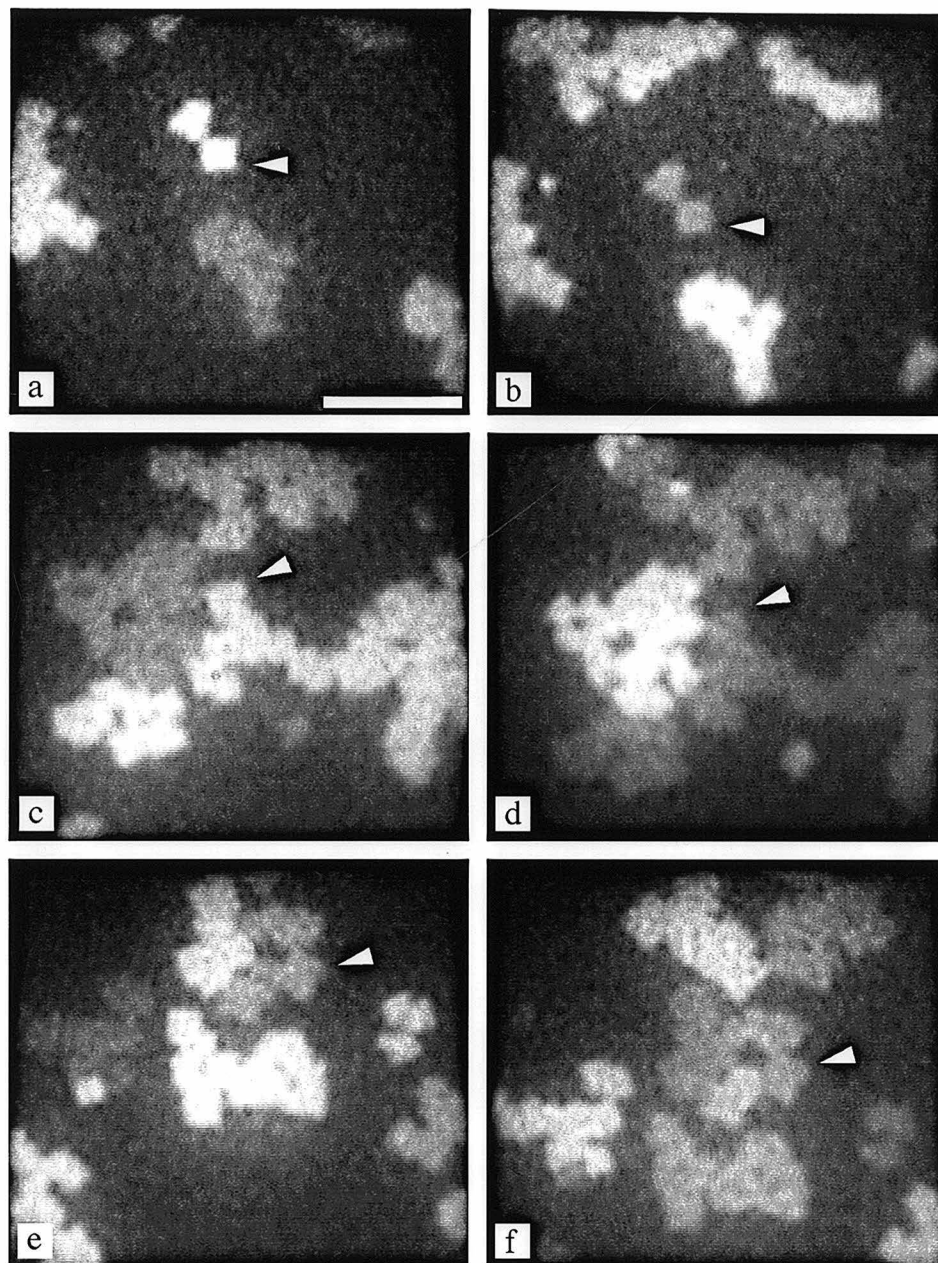


Figure 4.10. Fluorescence micrographs of streptavidin aggregates grown on DOIDA-Cu monolayer on buffered subphase (20 mM MOPS, 250 mM NaCl, pH 7.8) imaged through crossed polarizers. Images on the right and left were taken under perpendicular orientations of the polarizers. White arrows indicate equivalent points on the monolayer in each of the pairs of images. Scale bar = 100 μm .

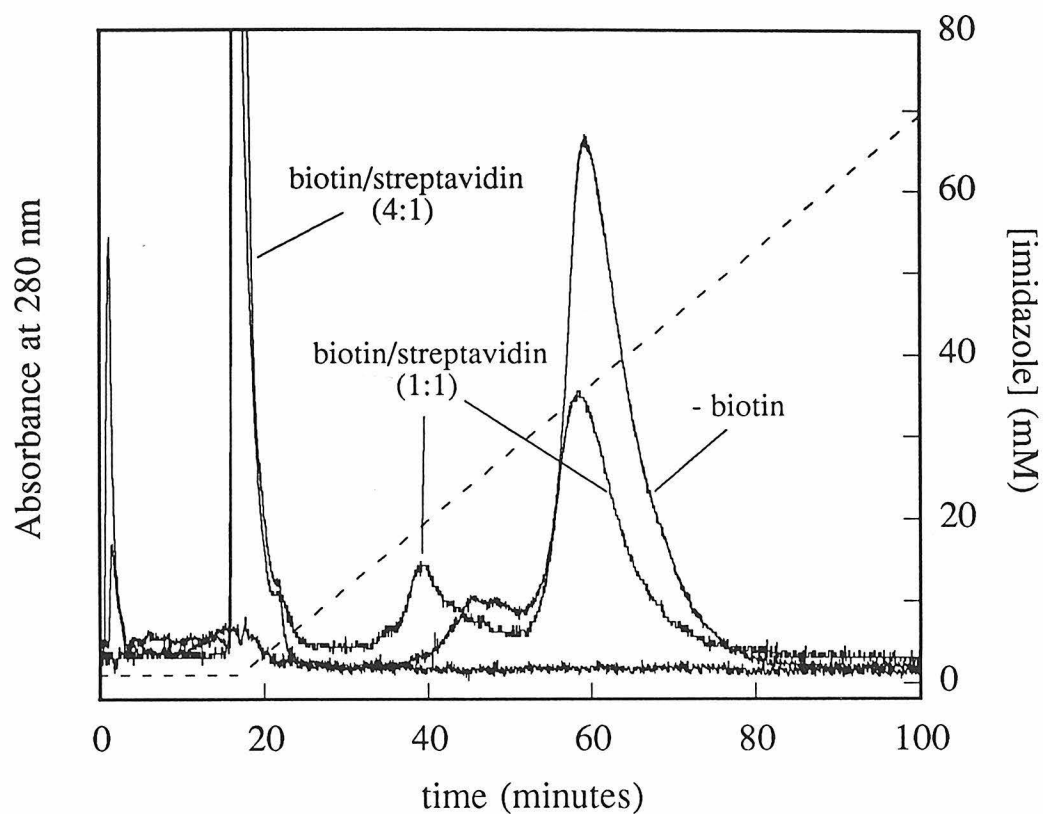


Figure 4.11. Chromatograms showing retention of streptavidin in the presence and absence of biotin, as indicated, in metal-affinity chromatography on an IDA-Cu-derivatized support. The protein was eluted with a linear gradient of imidazole (dotted line).

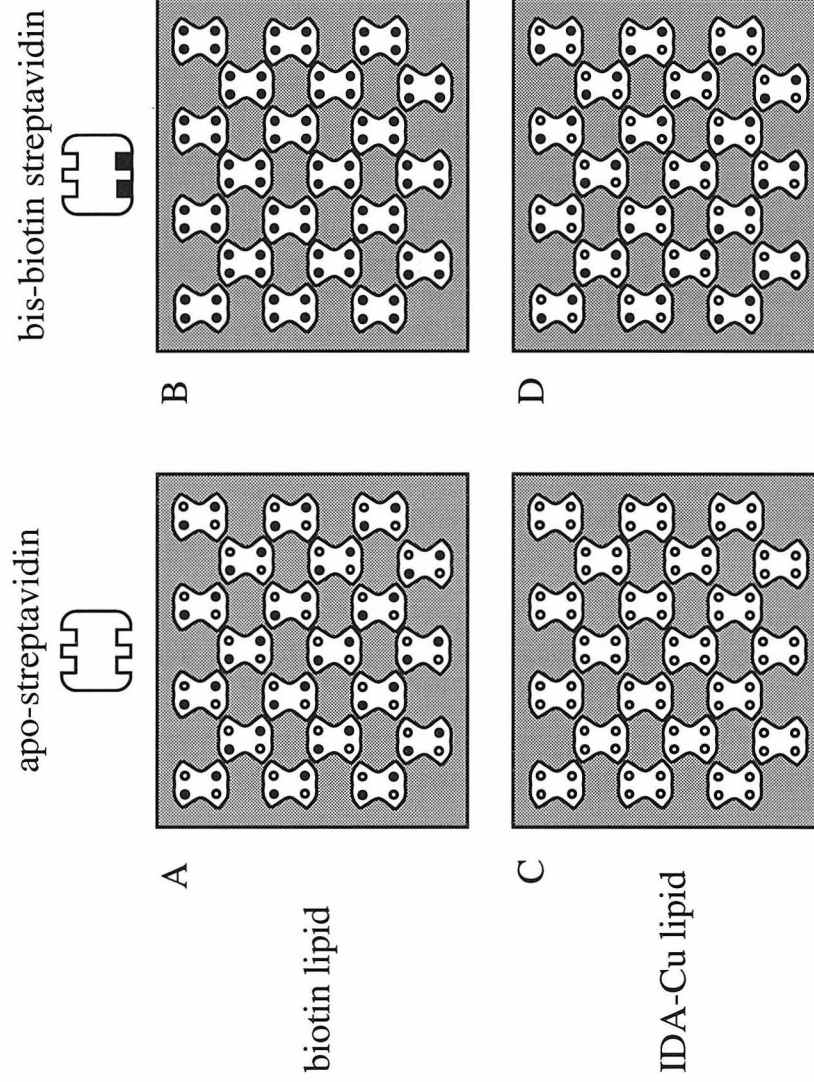


Figure 4.12. Schematic representation of projection map of 2D streptavidin crystals grown on biotin lipid and IDA-Cu lipid monolayers. The protein orientation, and therefore the crystal packing, are expected to be the same for streptavidin crystals grown on IDA-Cu lipid monolayers. Filled and unfilled circles represent biotin binding pockets filled with biotin and unfilled, respectively, for a biotin lipid-bound crystal. (A) and (D) Asymmetric protein-protein contacts produce H-shaped crystals. (B) and (C) Symmetric protein-protein contacts produce rectangular crystals.

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Chapter 5

**Design and construction of a UV-vis fluorescence microphotometer
for quantitation of fluorescent Langmuir monolayers.**

Introduction

Biological membranes are dynamic structures responsible for performing a wide variety of cellular functions. Frequently, membrane functions are controlled by extracellular signals. For instance, binding of external ligands by membrane-bound receptors (proteins or lipids) often induces reorganization or assembly of the receptor molecules. Formation of receptor aggregates leads to functions such as transmembrane signaling [1], endocytosis [2], thrombosis [3] and apoptosis [4, 5]. Synthetic membrane systems which seek to mimic biological membrane function have led to applications in sensing [6], functional molecular devices [7] and controlling the molecular architecture of new materials [8, 9]. To date, most examples of this approach have not taken advantage of the dynamic nature of lipid membranes. We are attempting to create simple, robust materials capable of reproducing important aspects of targeting, reorganization and assembly for producing functional materials.

We have chosen to use pyrene-containing membrane components as probes of ligand binding-induced membrane reorganization. The fluorescent properties of pyrene depend very strongly on its environment. For instance, pyrene emits as a monomer with a maximum at 377 nm. When an excited state pyrene molecule collides with a ground state pyrene, however, it forms an excited dimer species, or excimer, which emits as a broad, featureless band with a maximum at 470 nm (Figure 5.1) [10]. The ratio of excimer to monomer species depends on the rate of collision between pyrene moieties and is, therefore, a function of the local pyrene concentration and its lateral diffusivity in the membrane [11, 12]; an increase in either parameter leads to an increase in the ratio of excimer and monomer species. By measuring pyrene excimer and monomer intensities and changes in the ratio of intensities (E/M) upon introduction of an external ligand, ligand-induced membrane reorganization can be studied. Many investigators have used this approach to study interactions of proteins with pyrene-labeled lipid membranes [12-16].

Furthermore, a number of studies have used pyrene lipids to investigate ligand-induced membrane reorganization [17-20].

We have previously used pyrene lipid reorganization induced by binding of external ligands to produce functional materials (c.f. Chapter 1 and [21]). Metal ions were found to induce lipid reorganization in small, unilamellar vesicles consisting of 5% PSIDA (1-octadecyl-2-(9-(1-pyrene)nonyl)-*rac*-glycero-3-(8-(3,6-dioxy)octyl-1-amino-N,N-diacetic acid); c.f. Chapter 2) and 95% distearoyl phosphatidylcholine (DSPC). In the absence of metal ions, the pyrene fluorescence exhibited a large E/M ratio due to the presence of lipid aggregates of high local PSIDA concentration. Addition of divalent metal ions essentially instantaneously increased the monomer population at the expense of the excimer population. This dramatic decrease in the E/M ratio is believed to be due to dispersal of the pyrene lipid aggregates into the DSPC matrix upon binding of the metal ion by the IDA lipid headgroup. Although this example relies on disassembly, as opposed to assembly, of membrane components, the concept of ligand-induced membrane reorganization is apparent. This system is of practical interest as a simple, selective, and surprisingly sensitive fluorescent metal ion sensor. The sensitivity of the response to different transition metal ions generally parallels the known binding constants of the metals to IDA [22]. The sensor is most sensitive to copper, allowing detection at parts-per-trillion Cu^{2+} in solution.

PSIDA-Cu can target proteins and peptides to membranes *via* IDA-Cu-histidine complexation (c.f. Chapters 3 and 4 and [23]). Furthermore, membrane reorganization and patterning induced by multihistidine ligands may be investigated using the fluorescent properties of this lipid [24]. A UV-vis fluorescence microphotometer for quantitating pyrene fluorescence from Langmuir monolayer membranes *in situ* has therefore been constructed. In this chapter, I will describe the design of this instrument. As an example of the operation of the instrument, pyrene excimer and monomer fluorescence of mixed PSIDA/SOPC monolayers have been measured, and the resulting E/M ratios are used to estimate the diffusion coefficient of the membrane components. In addition, the

reorganization and cross-linking of PSIDA-Cu lipids by the multivalent ligand poly(histidine) were investigated.

Instrumentation

Overview of the UV-vis fluorescence microphotometer.

A schematic of the UV-vis fluorescence microphotometer is shown in Figure 5.2. An overview of the instrument, following the beam path through the system, will be presented prior to discussing the individual components. The instrument is based on an inverted epi-fluorescence microscope. A standard 100-watt Hg lamp serves as the excitation source. The mercury lamp provides efficient excitation of common fluorophores such as fluorescein and rhodamine, and emits a line at 334 nm suitable for pyrene excitation. An adjustable, luminous field diaphragm (LFD) controls the size of the illumination spot on the sample. The appropriate excitation wavelength is selected using a bandpass interference filter. The excitation light then strikes a dichroic mirror, which reflects light below a given cut-off wavelength and passes light above that wavelength, directing the beam toward the sample. We have employed a long-working-distance reflecting objective to focus the light onto the sample plane.

The resulting fluorescence emission is collected through the same objective and passes through the dichroic mirror. Again, a bandpass interference filter in the emission path is used to select the desired portion of the emission; wide band filters are used for imaging applications, while narrow band filters (± 5 nm) are employed for photon counting. From this point, the beam may follow several different paths. The beam may be directed out of the microscope, through a side port, to an intensified charged-couple device (CCD) camera for imaging. The images may be viewed on the video monitor, recorded to video tape, or digitized directly by a computer. Alternatively, the emission may be viewed through the microscope eyepieces. Finally, the emission light may be passed through the

top of the microscope for quantitation. Once exiting the microscope, the light first passes the planar field diaphragm (PFD) which limits the depth of field, ensuring the light reaching the photomultiplier tube (PMT) originated from the sample plane (*vide infra*). A lens then focuses the light onto the face of the PMT. The PMT is placed in a dry ice-cooled housing (-70°C) in order to limit dark current (random noise emitted from the PMT in the absence of any light impinging on the photocathode; dark current generally increases with increasing sensitivity of the photocathode) and increase the signal-to-noise ratio.

The photocurrent from the PMT anode consists of a series of variable, low-amplitude pulses, each of which corresponds to one photon impinging upon the PMT. In route to the pre-amplifier, a tee in the signal cable routes part of the signal to a snubber network which improves the pulse shape (*vide infra*). The four-stage pre-amp passes the signal on to the photon-counting device which serves as an amplifier, discriminator, and counter. The photon count is integrated over a given period of time, and the resulting count rate is acquired and stored on the PC.

Several other components of this system warrant mention. A variable bias, 3 kV power supply provides power to the photomultiplier tube. An automatic shutter is placed immediately adjacent to the PMT housing in order to protect the delicate PMT from stray light. The shutter may be controlled manually or may be controlled by the photon counter such that the shutter is open only when the counter is active. A heating element, with its own power supply, warms the window in front of the PMT to inhibit condensation due to the low operating temperature (-70°C). Finally, the entire apparatus is built on a vibration isolation table, and a metal framework supports the rather heavy PMT housing above the microscope.

Epi-fluorescence microscope.

The inverted epi-fluorescence microscope (Zeiss Axiovert 35) is a versatile platform for construction of the photometer. The inverted configuration allows the Langmuir

monolayer instrument to be placed directly above the stage of the microscope, with room above the trough for the Wilhelmy balance and for manipulation of the instrument (cleaning, filling, lipid spreading, etc.). The standard filter cube holds two sets of emission and excitation filters. However, the excitation and emission filters are linked such that only one emission wavelength may be observed for each excitation wavelength. A custom-made filter slider, located below the filter cube, allows the use of two different emission filters with one excitation filter for easy measurement of pyrene monomer and excimer signals. Because pyrene excitation occurs in the UV, care was taken to ensure the maximum excitation energy is transmitted to the sample. Thus, the standard lamp condenser, which gathers and collimates the excitation beam, was replaced with a quartz version. In addition, a UV-transmitting objective lens is required. Note, due to the window in the bottom of the trough and ~5 mm of water separating the objective from the monolayer interface, a long working distance (~7 mm total) is also necessary. Quartz, long-working-distance objectives were not readily available at the time this instrument was constructed, and, while such objectives are now available, they are prohibitively expensive. Rather, we have employed a reflecting objective (Ealing Electro-Optics, 36x, numerical aperture = 0.5, 8 mm working distance). By using a set of mirrors in place of the usual glass lenses, these objectives pass light from the far UV to the near IR. The disadvantage of these objectives is their lack of correction (for spherical and chromatic aberration, etc.). This is of little consequence for photometry, and quality images can nonetheless be obtained (c.f. images in Chapters 2-4).

Imaging system.

An intensified CCD camera system (Hamamatsu C2400-60, 68) is incorporated into the instrument for imaging. The CCD is attached to a side port on the microscope body, and a movable mirror allows the beam to be easily directed to either the camera or the PMT. The intensified CCD provides a fast, sensitive camera with essentially no lag; low lag time

is important for imaging the often rapidly moving Langmuir monolayers. In addition, the variable-gain intensifier allows visualization of very low-light-level images. The disadvantage of this system is that some blooming occurs (i.e., a single pixel, when intensified, broadens into multiple pixels) at high intensifier gain, producing a grainy image. The images can be saved to video tape in real time. More importantly, a frame grabber card in the PC (Scion, AG-5) is capable of digitizing the images at a full 640x480 pixels resolution and 30 frames per second. In addition, the frame grabber will average up to 132 frames, greatly improving image resolution and contrast at low light levels.

Planar field diaphragm.

As mentioned above, the PFD plays an important role in photometry by limiting the depth of field of the “image” reaching the PMT. The PFD consists of an adjustable, centrable iris placed in a real image plane above the microscope. This iris must be matched exactly, in size and centration, to the image of the LFD on the sample. In this way, only light originating from the illuminated sample plane reaches the PMT (pseudo-confocal mode; c.f. Figure 5.3). The PFD housing (Zeiss, photometer head O) contains a small light bulb and a movable mirror which back-project an image of the PFD onto the sample plane. Thus, using a sufficiently reflective sample, the PFD and LFD images can be viewed through the eyepieces simultaneously (Figure 5.3). Two set screws in the housing allow the PFD image to be superimposed on the image of the LFD as required.

Photomultiplier tube.

For detection of the typically weak fluorescence, an 11-stage Quantacon photomultiplier (RCA C31034A) was chosen. The photocathode exhibits a broad spectral response (185 to 930 nm), and the tube is covered with an ultraviolet-transmitting window allowing detection of the pyrene monomer fluorescence. The risetime of the tube is 2.5 ns. The high cathode responsivity (81 mA/W at 860 nm) and gain (0.6×10^6) make detection of single photons possible. In addition, the tube exhibits low dark current at reduced

temperatures (30 counts per second at -30°C). The tube is mounted in a dry ice-cooled housing and is operated at -70°C . A variable-bias (0-3 kV), high-voltage power supply provides the supply voltage across the tube. The tube is typically operated at a bias of 2 kV, as this bias was found to give the best compromise between low noise and high signal pulse amplitude.

Snubber.

A snubbing network is placed at the PMT anode and performs four important functions. The snubber provides a dc-resistive path between the anode and ground, without which the dark current may charge the signal cable to a few hundred volts, possibly damaging the pre-amplifier. The risetime of the PMT output signal pulse is often much faster than the fall time, producing a tail on the signal. The snubber will greatly reduce the falltime, improving pulse pair resolution of the system. The snubber can be used to cancel ringing in the PMT signal, which can cause multiple counts from a single photon. Finally, the snubber helps terminate reflections from the input to the pre-amplifier. The snubber used here was constructed from a 12-inch piece of coaxial cable terminated in a $100\text{-}\Omega$ pot. Output signal pulses from the PMT are split, 50% going to the photon counter, 50% going to the snubber. With the snubber pot adjusted to $50\text{ }\Omega$, there is no reflection. However, at less than $50\text{ }\Omega$, some portion of the signal is inverted and reflected back toward the anode. In addition, the reflected signal is delayed by the round trip time in the snubber cable. Thus, the amount of the inverted reflection can be adjusted such that tailing is canceled. The PMT signal was analyzed on a 300-MHz oscilloscope, and the snubber pot was adjusted to $13.5\text{ }\Omega$ where the best pulse shape was found.

Pre-amplifier.

A fast, four-stage pre-amplifier (Stanford Research Systems, SR445) amplifies the PMT output pulse in route to the photon counter. Each channel provides a gain of five, and

up to three channels can be cascaded for gains of 5, 25 and 125. The output pulse amplitude from the PMT can be estimated from

$$\text{pulse height (mV)} \approx 4 \times \text{PMT gain (in millions)} / \text{PMT risetime (in ns)} \quad (1)$$

Thus, the pulse height in our system is approximately $4 \times 0.6 / 2.5 = 0.96$ mV. The photon counter, however, requires a signal pulse near 100 mV. Therefore, three channels of the pre-amp are cascaded to provide a signal amplitude of 125×0.96 mV = 120 mV.

Photon counter.

The PMT output signal is quantified using a gated photon counter (Stanford Research Systems, SR400) which is a versatile instrument combining two fast 300-MHz amplifiers, three separate discriminators, and three 200-MHz counters. The discriminators screen incoming pulses based on slope and amplitude, rejecting shallow pulses or pulses below a set amplitude, greatly improving signal-to-noise. A pulse-height analysis was performed by scanning the discriminators across their full range (from 0 to -300 mV), thereby generating a distribution of count rate as a function of pulse height (Figure 5.4). The optimum discriminator setting is at the minimum between the noise and single photon peaks, and for our system the optimum was found to be ca. -100 mV. Count rates of 100,000 Hz to 30 MHz are routinely encountered from pyrene lipid monolayers. The counter is typically set for a count period of one tenth of a second with a 0.9-second dwell time. Thus, one data point per second is obtained. The photon counter then passes the measured count rate to the data acquisition program through a serial connection to the PC.

Data acquisition.

During measurements of the pyrene E/M ratio, it is necessary to manually switch between pyrene excimer and monomer emission filters. Variations in lamp intensity, monolayer lipid density and efficiency of oxygen purging cause significant fluctuations in the measured count rate on the scale of several seconds to minutes (*vide infra*). Ideally,

one should alternate between excimer and monomer intensities at each point, generating one E/M ratio every two seconds. This is impractical, however, as the instrument requires manual switching of the excimer and monomer filters. Therefore, an accurate measurement of the E/M ratio requires averaging the individual excimer and monomer intensities over a short time period (10 sec), taking the ratio of consecutive pairs of averages, and finally averaging the ratios over a longer period of time (3 min) (i.e., as opposed to averaging the excimer and monomer over a long period of time and taking one ratio). This procedure produces a large number of data points, however, and intensive manipulation of the data is required to produce a single, averaged E/M ratio; e.g., 180 total data points, 18 averages of 10 points each, and 9 ratios which are averaged to give the final E/M. Over the course of one experiment, ten E/M ratios may be required, making manual data manipulation impractical. A computer program has, therefore, been developed to perform much of the data manipulation (see Appendix). The program reads in the entire set of data (e.g., 1800 points for 10 E/M ratios) and sorts them into excimer and monomer counts based on user defined ranges. For each subset of points (e.g., 10 excimer counts), an average and variance are determined. The program then writes the averages and variances, in sets of nine pairs of excimer and monomer intensities, to a file which can easily be imported into a spreadsheet program for determination of the E/M ratio.

Results

The UV-vis fluorescence microphotometer has been used for measuring the ratio of excimer and monomer fluorescence intensities of pyrene lipid monolayers at the Ar-buffer interface under a variety of conditions. As described above, pyrene lipids in membranes emit fluorescence as monomers with a maximum at 377 nm, while the excimer species emits as a broad, featureless band centered at 470 nm (Figure 5.1). Two custom-made, narrow-band (10 nm FWHM) optical interference filters centered at these wavelengths were

used to isolate the monomer and excimer intensities. The filters exhibited maximum transmittances of 37% and 72% for the monomer and excimer filters, respectively.

Pyrene fluorescence is known to be very effectively quenched by oxygen [25, 26]. Therefore, the entire Langmuir instrument was placed inside a Plexiglas box, and the atmosphere within the box was purged with Ar. The effect of Ar flow into the box on total pyrene fluorescence of a mixed 5% PSIDA-Cu/95% 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) monolayer at 25 mN/m is demonstrated in Figure 5.5. With a sufficient flow of Ar into the box, the pyrene signal more than doubles. Approximately 15 minutes were required for the maximum removal of oxygen to be obtained. However, upon opening the box to the atmosphere, the fluorescence intensity returned to its original value within 60 seconds. Note the relatively large fluctuations ($\pm 8\%$) in fluorescence intensity. This noise is most likely the result of inhomogeneous purging of oxygen and vibrations of the monolayer surface due to the gas flow which cause the monolayer to move in and out of focus.

Sample data: E/M as a function of surface pressure.

As a demonstration of the operation of the fluorescence microphotometer, pyrene E/M ratios were measured as a function of monolayer compression for 5% PSIDA-Cu/95% SOPC mixed monolayers. These lipids are known to be well mixed at this composition and at all surface pressures (see Chapter 2). Plots of excimer and monomer intensities and E/M ratios versus molecular area are shown in Figure 5.6. The measured excimer intensity was found to be more than twice the monomer intensity at $117 \text{ \AA}^2/\text{molecule}$ ($\pi = 1 \text{ mN/m}$). (Note that the absolute intensities have not been corrected for varying transmittance of the optical components in the microscope, nor for the relative response of the PMT at different wavelengths. Therefore, the intensities and E/M ratios are system dependent quantities and should not be compared directly to results obtained from other experimental systems. One can correct the E/M ratio for these effects by multiplying by a factor of 0.3, allowing

comparisons to E/M ratios reported elsewhere.) As the monolayer was compressed, the excimer and monomer emissions increased steadily with decreasing molecular area. However, the excimer intensity increased more rapidly than the monomer intensity, and the E/M ratio therefore increased steadily. In fact, the E/M ratio increased by greater than a factor of two upon compression from 117 to 53 Å²/molecule (1 to 25 mN/m).

Poly(histidine)-induced monolayer reorganization.

As a further test of the instrument, the effect of the multivalent ligand poly(histidine) (poly(His)) on E/M ratios of 5% PSIDA-Cu/95% SOPC monolayers at 25 mN/m has been determined. The poly(His) concentration in the subphase below the monolayer was varied across six orders of magnitude, from 10⁻¹¹ to 10⁻⁶ M. For each concentration of poly(His), it was necessary to measure the change in E/M ratio as a function of time after injection of the peptide, as described above. An example of the raw data obtained for one particular poly(His) concentration is shown in Figure 5.7. Notice that each time point consists of nine sets of excimer and monomer counts, each containing 10 individual data points (see Figure 5.8). Calculating the averages and standard deviations for each set of points leads to a plot of fluorescence intensities and E/M as a function of time, a typical example of which is shown in Figure 5.9. In this particular experiment, the monomer intensity increased only slightly with time after injection of poly(His), while the excimer intensity increased 2.5-fold over 3 hours. The resulting E/M ratios, therefore, slightly more than doubled in the same time period.

Three such experiments were performed at each poly(His) concentration. The E/M ratio measured at 180 minutes after peptide injection was taken as the final E/M ratio in each experiment. This final E/M ratio was normalized by the initial E/M ratio before injection of poly(His) [(E/M)₀] to account for uncontrollable fluctuations in system parameters such as the lamp intensity, PMT temperature, etc. Plots of (E/M)/(E/M)₀ are shown in Figure 5.10. Values (E/M)/(E/M)₀ increased steadily with increasing poly(His) in the subphase due to

aggregation of the PSIDA-Cu lipids by the multivalent peptide. On the log-scale plot, the shape is roughly sigmoidal. In the absence of copper in the lipid headgroups, there was no increase in $(E/M)/(E/M)_0$ values at 10^{-7} M poly(His), indicating the lipid reorganization depends on coordination of the metal by the peptide.

Discussion

A UV-vis photometer based on a commercial fluorescence microscope has been constructed for quantitation of fluorescence from Langmuir lipid monolayers. The instrument is capable of single-photon counting, and count rates as low as 10,000 Hz can be measured. Various optical components of the microscope, including the objective, were customized to allow UV excitation and quantitation. Improvements in sensitivity, signal-to-noise ratio and image quality could be achieved by addition of a nitrogen laser operating at 334 nm as the excitation source and a higher numerical aperture objective to provide more efficient collection of the fluorescence. Because the instrument is based on a standard fluorescence microscope, photometry and imaging experiments can be performed simultaneously. Further, the instrument was designed to be highly flexible. For instance, with the addition of a laser light source and an appropriate sample holder, the photometer could be used as a total internal reflection fluorescence microscope (TIRFM) [27, 28]. Similarly, addition of a high-power laser source and an acousto-optic modulator would result in an instrument suitable for fluorescence-recovery-after-photobleaching (FRAP) measurements of lateral membrane diffusion [29, 30].

Fluorescence microscopy using UV excitation or emission is far less common than fluorescence microscopy at visible wavelengths. However, a few UV fluorescent probes exhibit unique properties which make them highly useful for probing the physico-chemical properties of their environment such as membrane dynamics or polarity. Thus, UV fluorescence microscopy has occasionally been employed to gather information which may not be accessible from any other type of experiment. For instance, the excimer-forming

properties of pyrene have been utilized to probe the dynamics of cell membranes *in vivo* [31]. Pyrene-containing lipids were also used to image Ca^{2+} -induced phase separation in lipid monolayers [32]. In addition, uptake and localization of the common environmental pollutant benzo(a)pyrene by living cultured cells was visualized by fluorescence microscopy of the pollutant itself [33].

There have been various reports in the literature of instruments capable of quantitation of pyrene fluorescence from thin samples such as Langmuir monolayers [25, 34-36]. Very often in these systems, excitation is delivered to and emission is collected from the interface through bundled optical fibers. Similarly to our instrument, photon counting techniques are typically used for detection. Such instruments have the advantages of not requiring a microscope, and the excitation or emission (or both) may be passed through a monochromator, allowing for fluorescence spectra to be acquired. In addition, the macroscopic sample size produces a large, easily detected signal. The disadvantages of these systems are that no imaging of the sample is possible and they are limited to simple photometry; i.e., applications such as TIRFM would not be possible. In addition, such instruments are limited to macroscopic (at least in two dimensions) samples. Our instrument, in contrast, can be used to investigate microscopic samples; e.g., fluorescence within lipid domains could be discriminated from fluorescence from without. Perhaps the most sophisticated instrument described to date, however, is that of Merkel and Sackmann [35]. Their instrument, capable of imaging microscopy of pyrene, utilized a nitrogen laser as excitation source allowing images of monolayers containing as little as 0.5 mol% pyrene lipids. A monochromator in the emission path allowed selection of excimer or monomer wavelengths. Finally, the relatively long lifetime of the pyrene probe allowed use of a digital storage oscilloscope as data acquisition system and, therefore, time-resolved measurements for determination of fluorescence lifetimes were possible.

Quantitative fluorescence microscopy is complicated by a number of factors. The amount of fluorescence detected is affected by the properties of the illumination source, the

optical and spectroscopic properties of the microscope, and the resolution, sensitivity, and signal-to-noise properties of the detector. In addition, non-uniform fluorophore concentrations, self-quenching at high fluorophore concentrations, photo-bleaching and variation in the physical environment can all effect the fluorescence quantitation. Many of these confounding factors can be overcome by ratio detection, since many of these factors have a similar effect on intensities at different wavelengths. Fortunately, for pyrene fluorescence, the relevant quantity is usually the E/M ratio, and these various factors are controlled for inherently.

Determination of lateral diffusion coefficient from pyrene E/M.

In the 5% PSIDA-Cu/95% SOPC lipid monolayer, the E/M ratio increased steadily from 2.5 at a surface pressure of 1 mN/m ($117 \text{ \AA}^2/\text{molecule}$) to 6.0 at 25 mN/m ($53 \text{ \AA}^2/\text{molecule}$). The individual excimer and monomer intensities also increased during compression. This may at first seem surprising, as an increase in the E/M ratio is often the result of a population inversion; i.e., an increase in the excimer fluorescence due to a decrease in the monomer fluorescence. Such a population inversion likely occurs during compression of the PSIDA-Cu/SOPC monolayer, as well. However, monolayer compression also results in an increase in the total lipid density which, in turn, causes an increase in total fluorescence intensity. There are simply more fluorophores present in the field of view at any given time. The increase in lipid density apparently counteracts the decrease in the fraction of the lipids emitting as the monomer species and enhances the emission from the excimer population. Similar changes in excimer and monomer fluorescence intensities during compression of mixed pyrene-containing monolayers have been reported [25, 34]. One can easily compensate for the decrease in area by normalizing the fluorescent intensities by the lipid density ($1/\text{molecular area}$).

Lateral membrane diffusion constants may be estimated from E/M ratios of pyrene lipids by several methods [11, 34, 37, 38]. In one such approach, the pyrene-containing

lipid is modeled as undergoing a random walk through the monolayer of the matrix lipids [11, 12]. This method assumes the pyrene lipid is homogeneously distributed throughout the membrane, and formation of excimers results from diffusion-controlled collision of the pyrene lipids. The diffusion coefficient can be written as a function of the frequency of collisions experienced by the lipids according to

$$D_{\text{diff}} = v_{\text{col}} \lambda / 4d_c c \quad (2)$$

where v_{col} is the number of collisions per second experienced by one molecule, λ is the length of one diffusional jump, d_c is the van der Waals diameter of the diffusing fluorescent lipid (i.e., how close two lipids must come to be considered a collision), and c is the two-dimensional concentration of the fluorescent lipid in the membrane. The collision frequency can be related to the E/M ratio to give

$$D_{\text{diff}} = \frac{E}{M} \frac{k_f}{k'_f} \frac{\lambda}{4d_c \tau'_0 c} \quad (3)$$

in which k_f and k'_f are the rate constants for monomer and excimer emission, respectively, and τ'_0 is the excimer fluorescence lifetime.

The value of k_f/k'_f is an intrinsic property of pyrene and can be estimated as 0.1 [11]. The van der Waals radius of the pyrene lipid must also be estimated, and in this analysis a value of 8 Å will be used. The excimer lifetime of PSIDA-Cu in SOPC liposomes has been measured as 40 ns [39], and this value is expected to apply to the monolayer membrane, as well. The length of one diffusional jump, λ , is assumed to be equal to one lattice spacing in the monolayer, and $\lambda = 2(A/\pi)^{1/2}$ where A is the mean molecular area of the monolayer. Finally, c is simply the inverse of the molecular area of

the lipids in the monolayer, A , multiplied by the mole fraction of pyrene-containing lipid, x_{PSIDA} . Equation (3) can now be written as

$$D_{\text{diff}} = \frac{E}{M} \frac{A^{3/2}}{6400\pi^{1/2}x_{\text{PSIDA}}} \quad (4)$$

Shown in Figure 5.11 are the E/M ratios and diffusion coefficients calculated from equation (4). The diffusion coefficients are on the order of 10^{-7} cm²/sec. This is in agreement with known diffusion coefficients for fluid phase lipid membranes at room temperature [34, 40, 41]. The diffusion coefficient decreases upon compression of the monolayer, as might be expected from the increased surface pressure. The change in the diffusion coefficient is not very large, however, decreasing by only 30% upon compression from 1 to 25 mN/m. This is also to be expected for a monolayer in the fluid phase [41].

E/M increase reflects binding of multivalent ligand.

Changes in the E/M ratio of the PSIDA-Cu lipid were used to observe reorganization of the membrane components upon binding to the multivalent ligand poly(His). The histidine homopolymer is a mixture of linear chains of molecular weight 11,000 to 14,000; each chain consists of 80 to 90 histidine monomers. Due to its multiple side chains, poly(His) can coordinate several IDA-Cu headgroups simultaneously. Thus, the polymer is expected to bind very strongly to the PSIDA-Cu-containing monolayer. In addition, due to the high local concentration of ligands, the multiple, simultaneous histidine-lipid interactions result in a local increase in the PSIDA-Cu concentration and “cross-linking” of neighboring lipids by the multivalent poly(His). The high local pyrene concentration and cross-linked lipids are reflected in an increase in the E/M ratio. The exact relation between the E/M ratio and (1) the amount of peptide bound to the monolayer and (2) the number of cross-linked ligands is complicated by the high degree of multivalency of poly(His) and, unfortunately, is not understood. Addition of poly(His) to the subphase beneath 5% PSIDA-Cu/95% SOPC monolayers to concentrations greater than or equal to

ca. 0.1 nM resulted in an increase in the E/M ratio. The E/M ratio increased steadily between 0.1 nM and 0.5 μ M, but appeared to saturate at concentrations greater than 0.5 μ M. Concentrations of poly(His) higher than 1 μ M were unattainable due to the limited solubility of the peptide at pH 7.5. Note, in the absence of copper, no change in the E/M ratio was observed even at 0.1 μ M poly(His), confirming the role of the histidine-metal coordination in the reorganization of the membrane.

Cross-linking of mobile membrane receptors by multivalent ligands has been modeled mathematically [42, 43]. According to this model, a distribution in the number of cross-linked receptors is observed with a maximum at a ligand concentration, L , equal to K_d/f , where K_d is the intrinsic dissociation constant for one ligand binding one receptor and f is the valency of the ligand. Increase in the ligand concentration beyond this value decreases the number of cross-links. This can be understood physically by imagining a membrane which is homogeneously covered with multivalent ligands (corresponding to a very high solution ligand concentration). In this situation, the membrane receptors “see” a homogeneous distribution of ligands, and there is no driving force for receptor aggregation or cross-linking. Examples of the distribution of the number of cross-links over a wide range of ligand concentrations are shown in Figure 5.12 for several values of the valency, f , from 2 to 80. Higher ligand valency moves the maximum toward lower ligand concentration and broadens the distribution on the low-ligand-concentration side of the maximum.

Because the E/M ratio appears to reflect the number of cross-linked lipids (albeit in a complicated fashion), we might expect the E/M ratio to go through a maximum at some poly(His) concentration in the subphase. No maximum was observed between 10^{-11} and 10^{-6} M, however, indicating either the above model does not describe our system, or the maximum occurs at higher poly(His) concentration than 1 μ M. Since the decrease in cross-linking predicted by the model begins near $L = K_d/f$, and assuming K_d is of the same order as for imidazole binding to IDA-Cu, $10^{-3.5}$ M, we would expect cross-linking to begin

decreasing rapidly around $L = 4$ to $30\ \mu\text{M}$ ($f = 10$ to 80). Thus, it appears the maximum cross-linking is simply not accessible in our system due to the limited solubility of poly(His). Note that f is the number of ligands able to bind receptors simultaneously. While we know each poly(His) chain contains ~ 80 ligands, the number of those ligands able to simultaneously bind the lipids is unknown. While no fitting of the present data to this model is possible due to the unknown dependence of the E/M ratio on the amount of cross-linking, the shape of the E/M versus [poly(His)] plot is consistent with the distributions predicted by the model.

The formation of cross-linked PSIDA-Cu lipids by binding of poly(His) has also been imaged using fluorescence microscopy [24, 44]. As shown in Figure 5.13 A, addition of $150\ \text{nM}$ poly(histidine) to the subphase below 5% PSIDA-Cu/95% SOPC monolayers resulted in the appearance of circular spots (ca. $50\ \mu\text{m}$ diameter) of very bright pyrene excimer fluorescence, corresponding to regions of high local pyrene concentration. Furthermore, the rhodamine-labeled poly(histidine) was localized to these same regions (Figure 5.13 B). (It should be noted that the two spots in Figure 5.13 A and B are, in fact, a single spot. Because the monolayer is fluid, the spot moves some $100\ \mu\text{m}$ during the time required to change from the pyrene filter set to the rhodamine filter set (ca. 1-2 sec).)

One should note that these poly(His) domains appear on the monolayer in addition to the overall increase in the E/M ratio. The flow of Ar in the enclosure around the monolayer causes relatively rapid convection of the monolayer at the interface. This convection ensures that the measured excimer and monomer intensities are averages over large areas of the monolayer. From the images in Figure 5.13, it appears the E/M ratio within the domains is considerably higher than the surrounding phase. Note, also, that the size of the domains indicates multiple poly(His) chains are aggregated in each domain. Such aggregation could be the result of the relatively high concentration of poly(His) at the interface and the limited solubility of poly(His) at pH 7.5.

Conclusion

Reorganization and assembly of membrane proteins effected by binding of external ligands are an integral part of many cellular functions. Synthetic mimics of biological membranes form the basis for materials for a wide variety of applications. We are attempting to mimic biological ligand-induced membrane assembly in order to produce new functional materials. We have chosen to use Langmuir monolayers as the basis of our membrane mimetic materials, and the pyrene-labeled lipid, PSIDA-Cu, optically signals reorganization due to binding of multivalent ligands. A UV-vis fluorescence microphotometer has, therefore, been built. The photometer, based on an inverted fluorescence microscope and photon-counting instrumentation, is capable of sensitively quantitating fluorescence from Langmuir monolayers. The design of the instrument is very versatile and, with minor additions, could be transformed into other optical instruments such as a TIRFM or FRAP apparatus. The utility of the photometer for quantitating PSIDA monolayer fluorescence has been demonstrated. A diffusion coefficient for mixed PSIDA-Cu/SOPC monolayers of $1\text{--}2 \times 10^{-7} \text{ cm}^2/\text{sec}$ was estimated based on PSIDA-Cu E/M ratios. In addition, cross-linking of PSIDA-Cu by poly(histidine), analogous to biological ligand-induced receptor organization, was observed by changes in the excimer and monomer intensities and the E/M ratio.

Experimental

Measurement of E/M during monolayer compression.

Monolayers were prepared on a computer-controlled Langmuir instrument (KSV Instruments, model M1200) with a surface area of $250 \times 75 \text{ mm}^2$. The entire film balance was placed inside a Plexiglas box, and the atmosphere inside the box was purged with Ar. The surface pressure was measured by the Wilhelmy plate method, using plates cut from filter paper (Whatman, No. 1). The plate was rinsed thoroughly with chloroform and

calibrated with stearic acid prior to use. For all experiments, the subphase was buffered with 20 mM MOPS, 100 mM NaCl at pH 7.5, and the temperature was maintained at 24 ± 0.5 °C. Lipid solutions were prepared in chloroform, and IDA lipid/SOPC mixtures were prepared by mixing appropriate volumes of stock solutions prior to spreading. The IDA lipids were pre-metalated by adding an aliquot of concentrated CuCl_2 in methanol to the lipid solutions. Cu^{2+} excess in the lipid solution is ~10-15%. Approximately 40 μL of the lipid solution were spread with a Hamilton syringe and, after allowing 15 minutes for solvent evaporation, the monolayer was compressed at a constant rate of $3 \text{ \AA}^2/\text{molecule}\cdot\text{min}$ to a surface pressure of 1 mN/m. After allowing ~10 minutes for equilibration of the monolayer, the excimer and monomer intensities were measured as described above. Compression was then resumed. Compression was stopped and the pyrene fluorescence was measured at surface pressures of 1, 5, 10, 15, 20 and 25 mN/m.

Measurement of poly(histidine)-induced changes in E/M.

Poly(histidine) experiments were performed in a Petri dish hydrophobized with octadecyltrichlorosilane. Holes cut in the lid of the Petri dish allowed access of the Wilhelmy plate and entrance of Ar. Surface pressure was again measured using plates cut from filter paper. Monolayers were spread dropwise until the desired surface pressure was attained. Time was allowed between drops for stabilization of the surface pressure. Approximately 60 minutes after spreading, a concentrated poly(His) solution was injected into the 15-mL subphase through the monolayer. The pyrene excimer and monomer intensities were measured immediately prior to, and at various times after, addition of poly(His). In all experiments, the E/M ratio had stopped changing 3 hours after peptide injection, and this time point was used as the “final” E/M. E/M measurements were made in triplicate for each poly(His) concentration in the subphase.

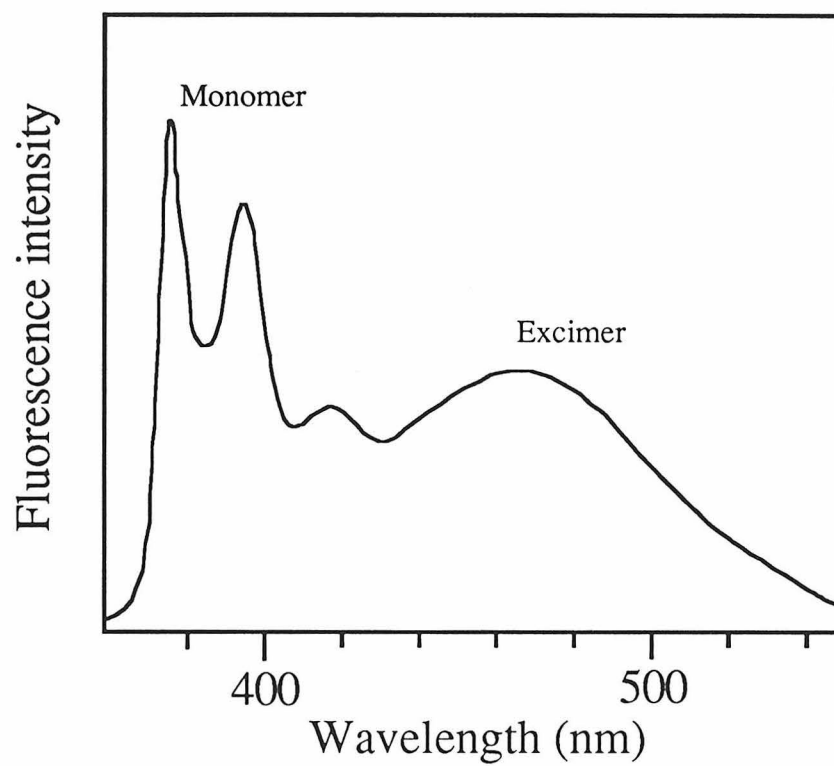


Figure 5.1. Representative steady-state fluorescence emission spectrum of 5% PSIDA in SOPC liposomes. Monomer peak at 377 nm and excimer peak at 470 nm are indicated.

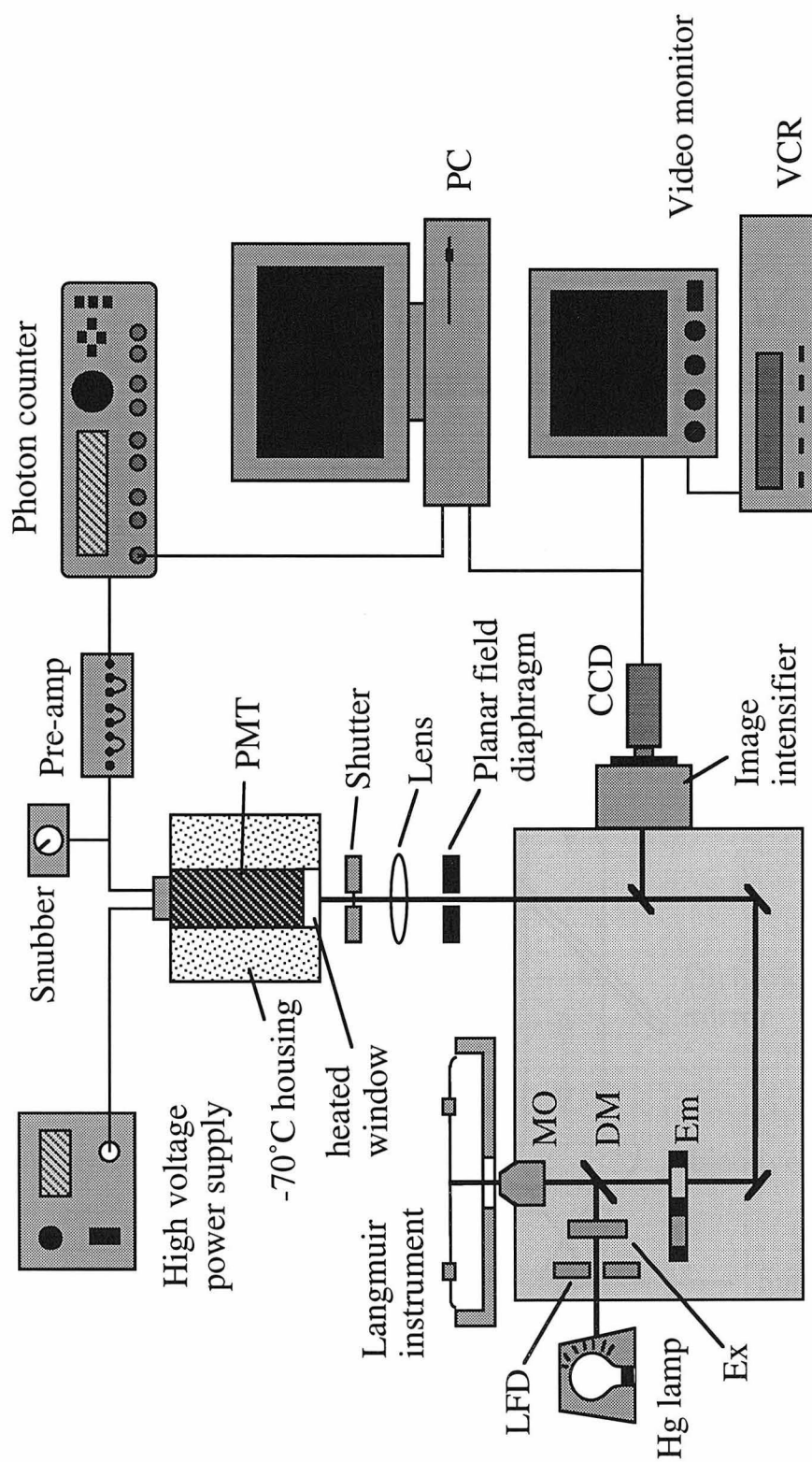


Figure 5.2. Schematic of UV-vis fluorescence microphotometer. Ex, excitation filter; LFD, luminous field diaphragm; Em, emission filter; DM, dichroic mirror; MO, microscope objective; CCD, charge-coupled device video camera; PMT, photomultiplier tube; VCR, video cassette recorder; PC, personal computer.

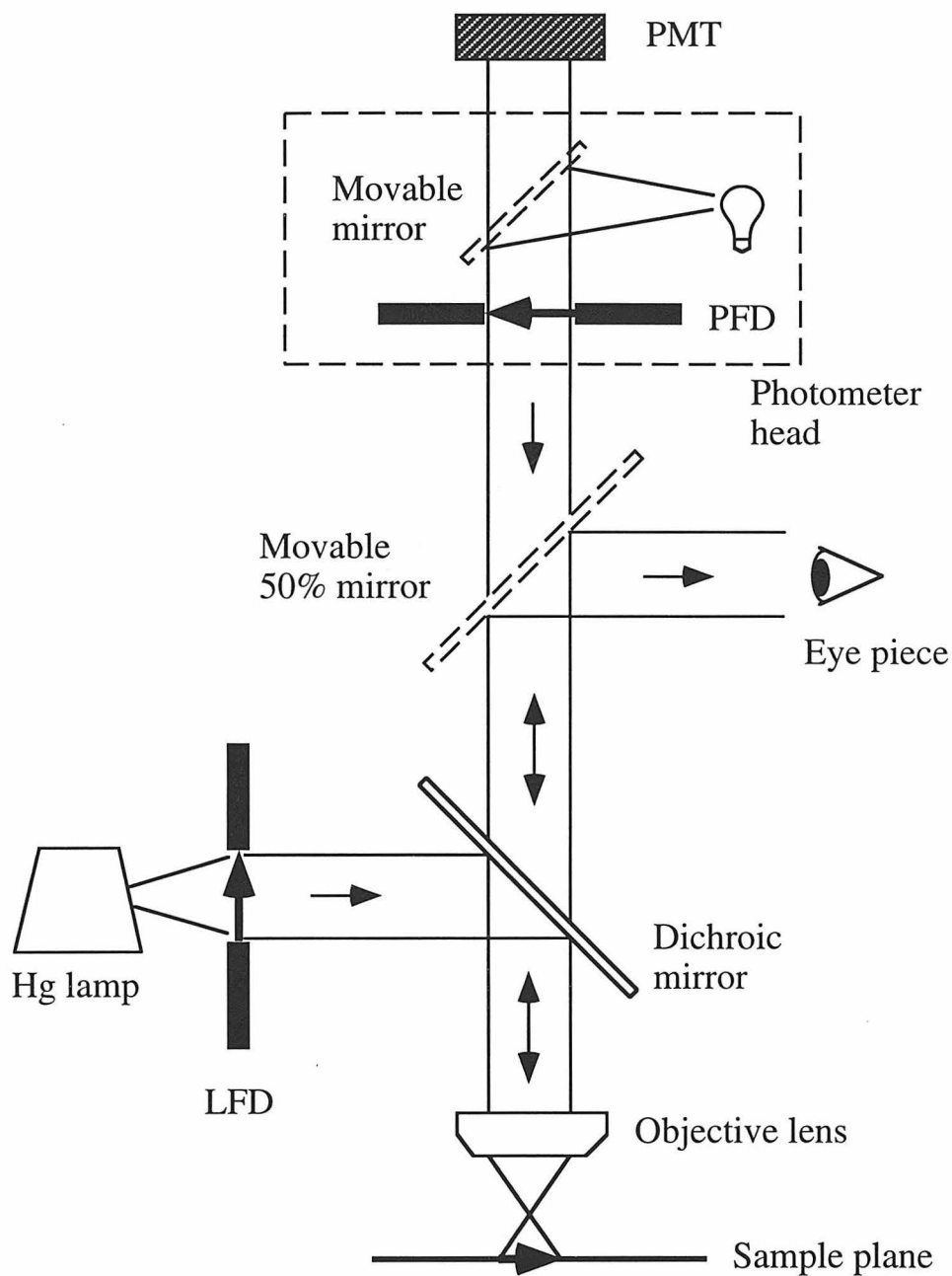


Figure 5.3. Optics allowing alignment of the planar field diaphragm (PFD) with the luminous field diaphragm (LFD). The Hg lamp casts an image of the LFD onto the sample. A lamp in the photometer head simultaneously casts an image of the PFD through the dichroic mirror and onto the sample. A sufficiently reflective sample allows both diaphragms to be viewed simultaneously through the eyepieces.

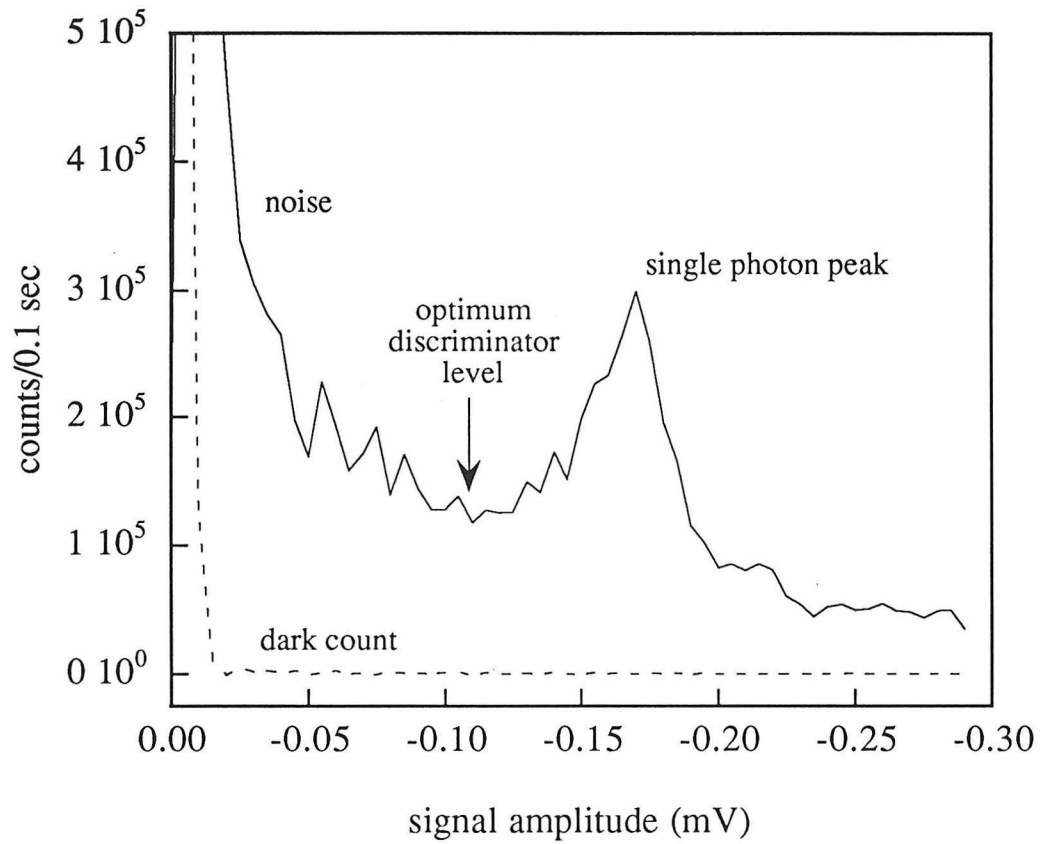


Figure 5.4. Pulse-height analysis of photomultiplier tube operated at 2 kV. The signal peak is centered at ca. -160 mV. Random electronic noise decays exponentially with increasing amplitude. The optimum discriminator setting is at the minimum between the signal and noise peaks, ca. -100 mV.

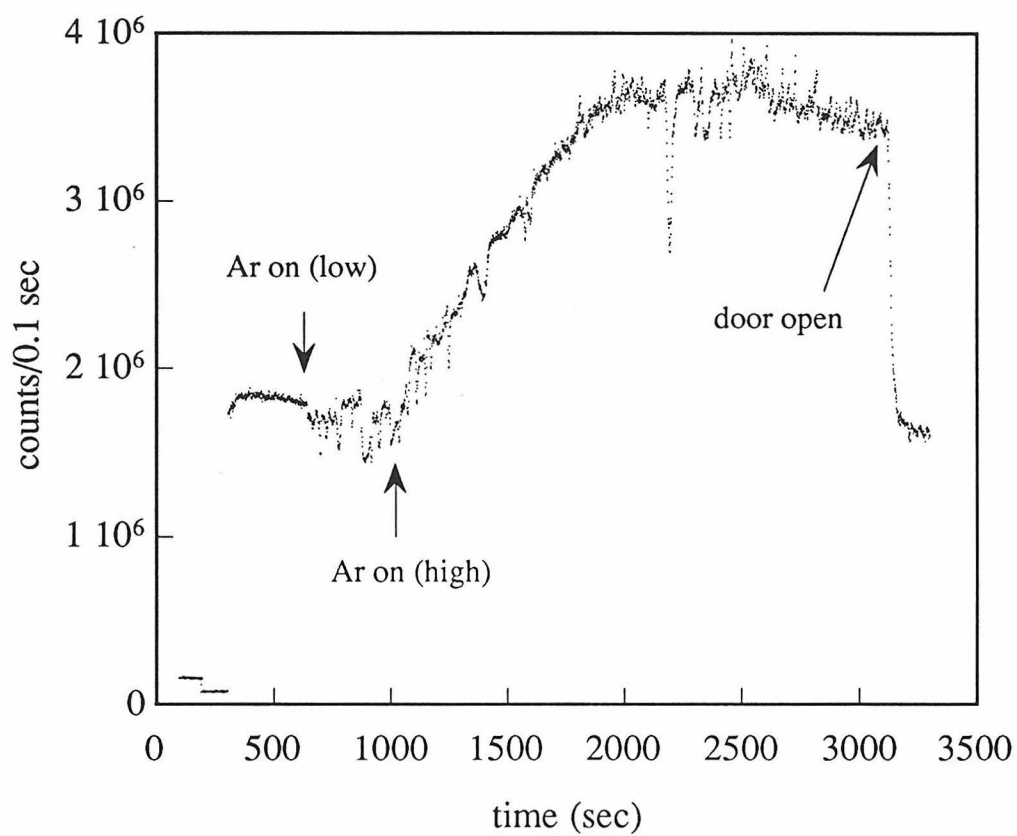


Figure 5.5. Effect of purging the atmosphere above the monolayer with Ar on PSIDA excimer fluorescence intensity. The presence of Ar approximately doubles the signal intensity within 15 minutes. Opening the door of the enclosure decreases the fluorescence to its original intensity within 60 seconds.

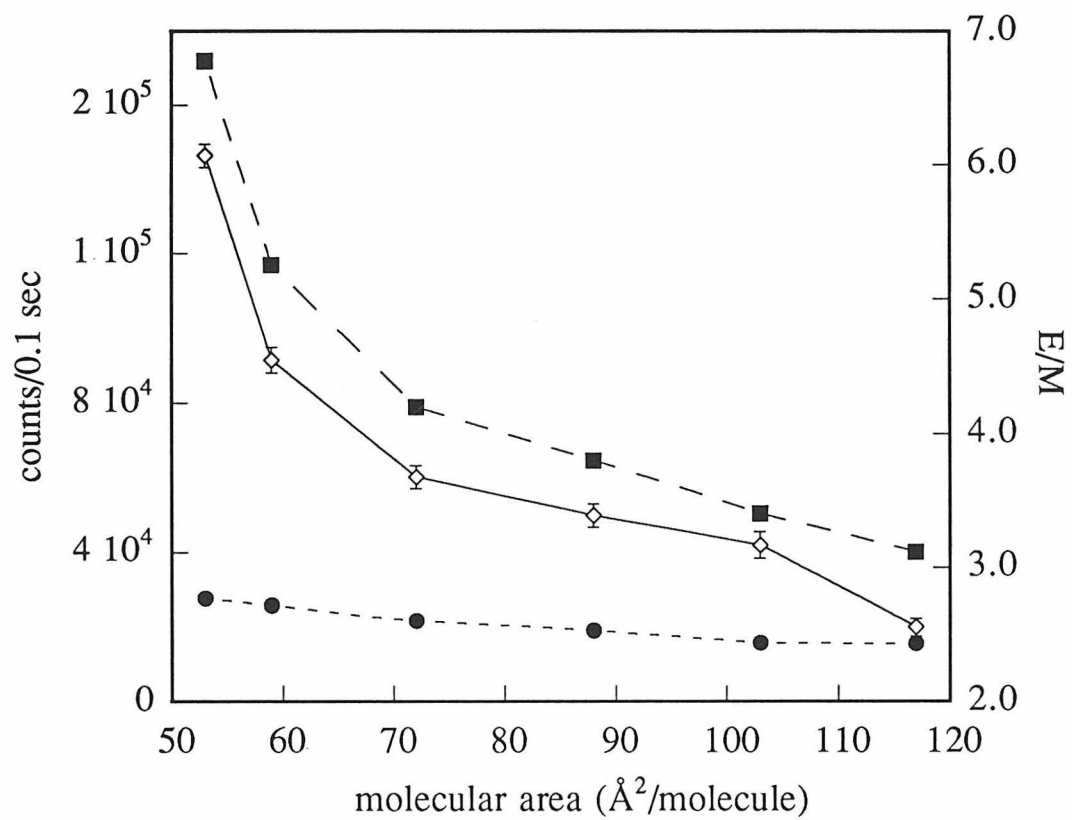


Figure 5.6. Excimer and monomer fluorescence intensities and E/M ratios during compression of 5% PSIDA-Cu/95% SOPC lipid monolayer (●, monomer; ■, excimer; ◇, E/M).

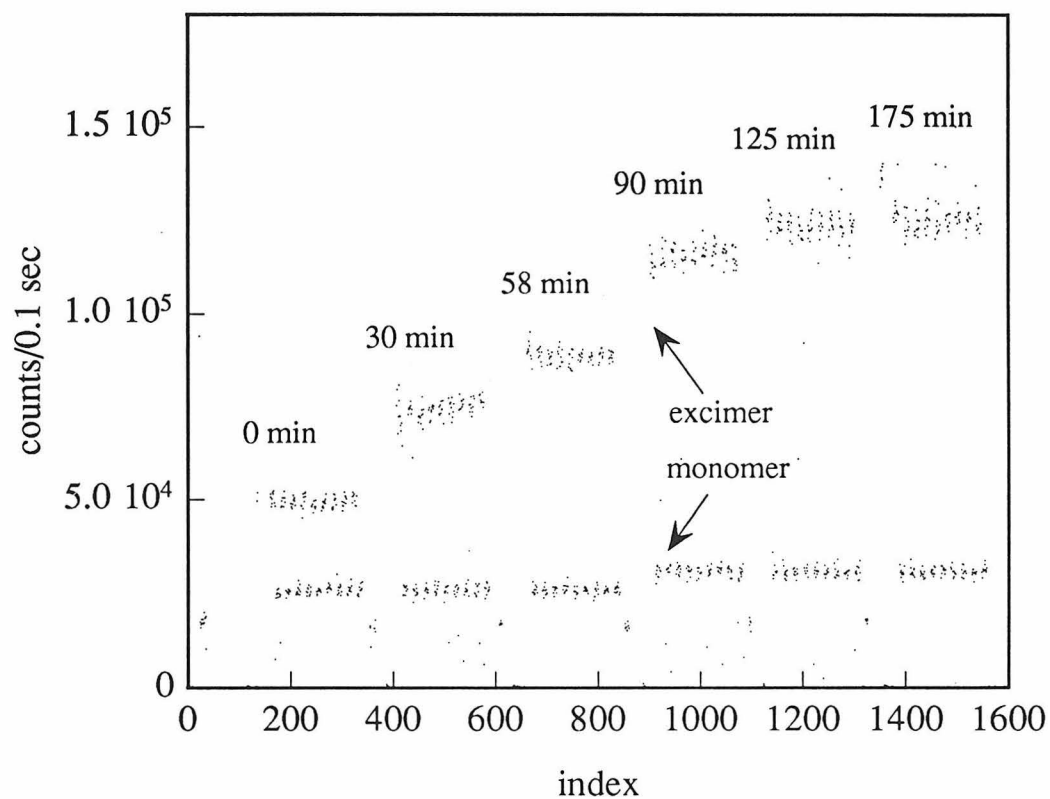


Figure 5.7. Example of raw data of E/M change upon injection of $1 \mu\text{M}$ poly(histidine) under 5% PSIDA-Cu/95% SOPC lipid monolayer. At each time, 9 sets of excimer and monomer intensities (10 points each) are obtained.

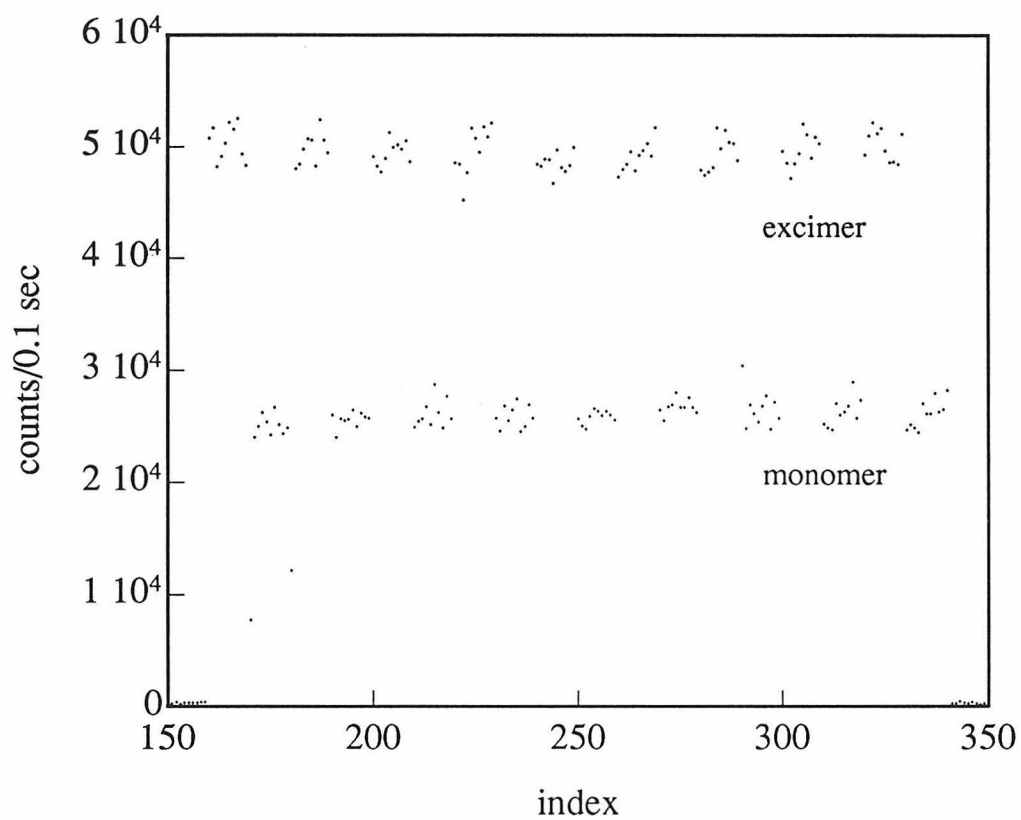


Figure 5.8. Expanded view of one time point from Figure 5.7. Each set of 9 points are averaged. Adjacent excimer and monomer intensities are divided to give E/M ratio. The nine ratios are averaged to give the final E/M.

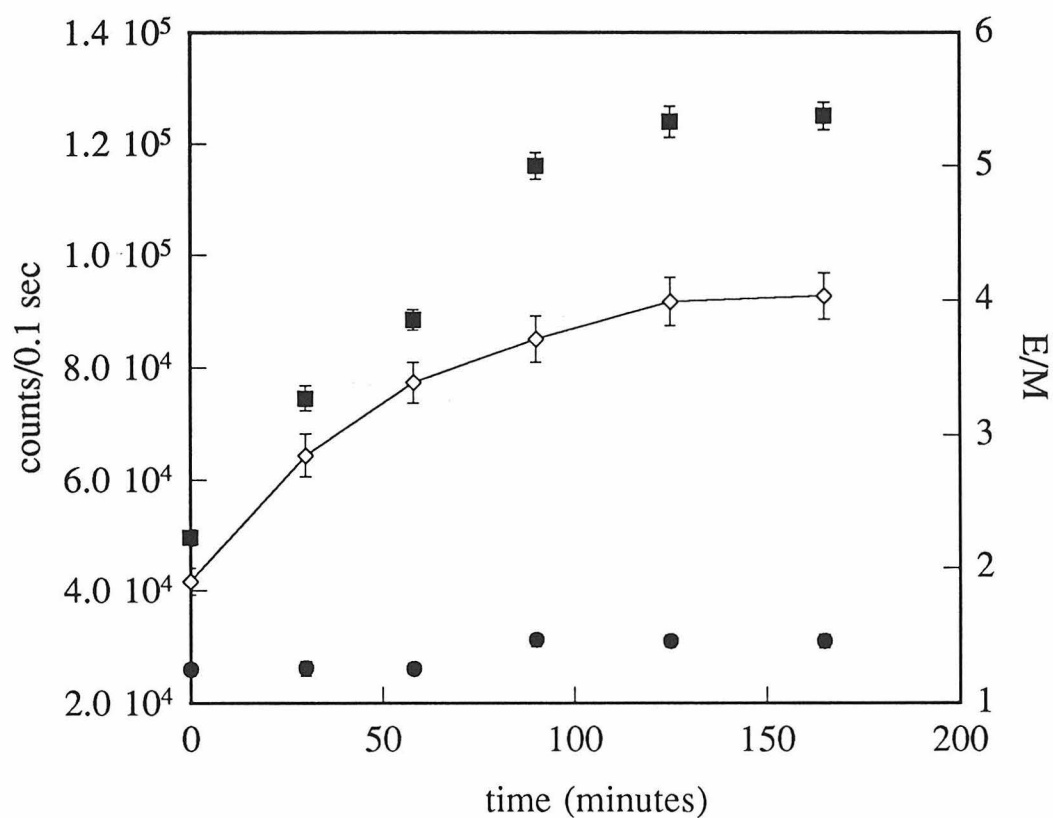


Figure 5.9. Excimer and monomer fluorescence intensities and E/M ratios versus time after injection of poly(histidine) into the subphase beneath 5% PSIDA-Cu/95% SOPC mixed monolayer (●, monomer; ■, excimer; ◇, E/M).

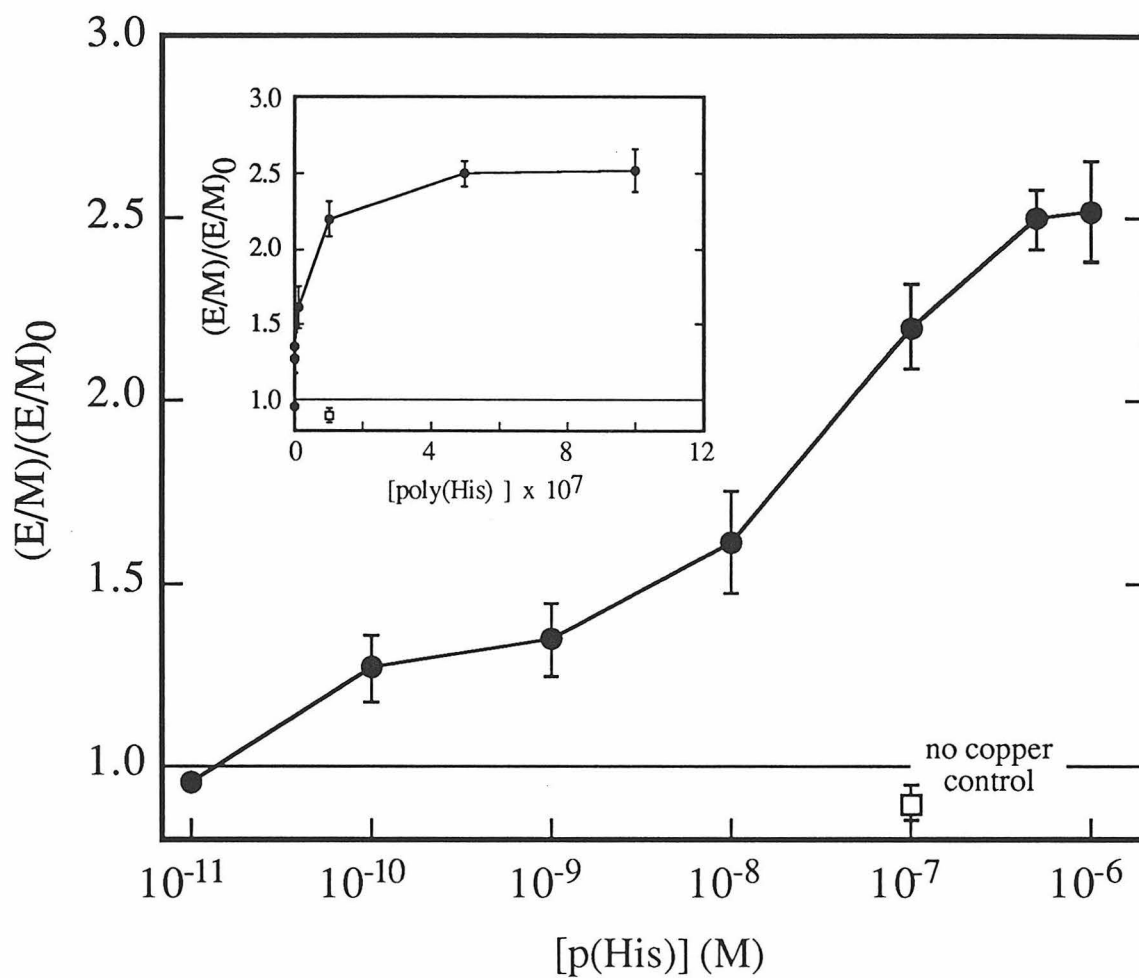


Figure 5.10. E/M as a function of poly(histidine) concentration for 5% PSIDA-Cu/95% SOPC mixed monolayers. Inset shows same data plotted on a linear scale of poly(histidine) concentration. Note, in the absence of copper, no change in E/M was observed.

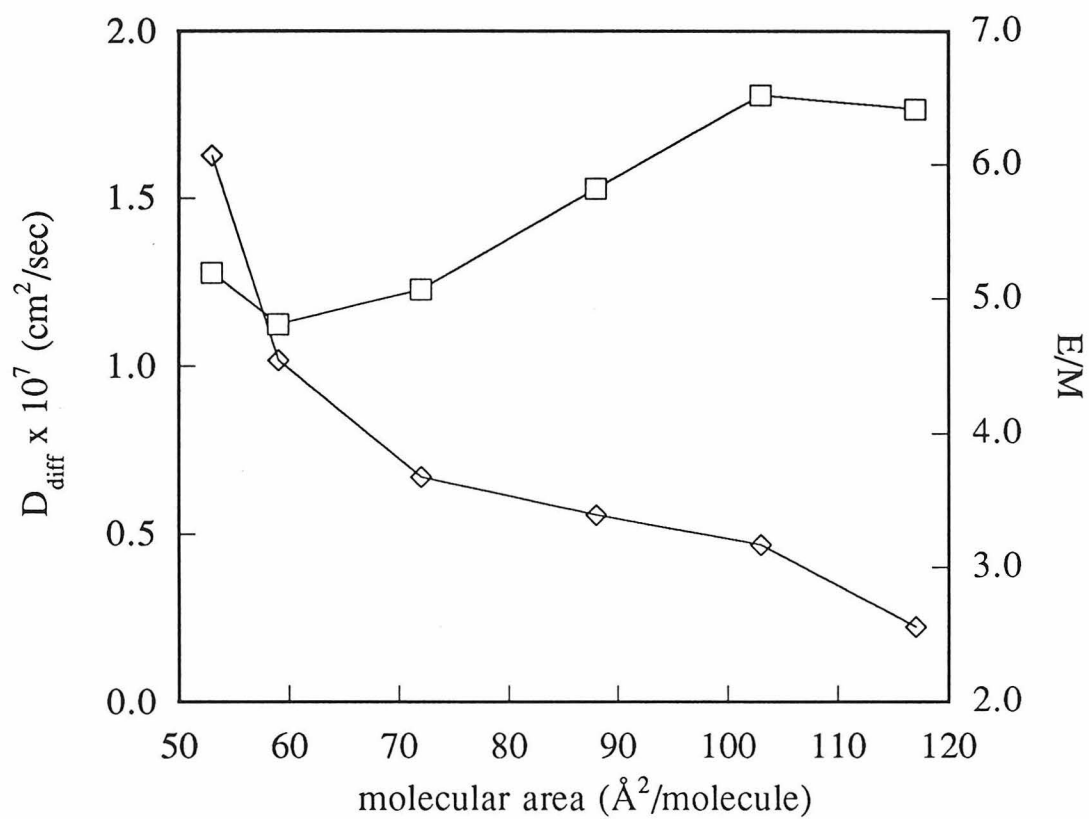


Figure 5.11. E/M ratios ($\diamond$) and estimated diffusivities (D_{diff} , $\square$) during compression of 5% PSIDA-Cu/95% SOPC lipid monolayers at the Ar-buffer interface.

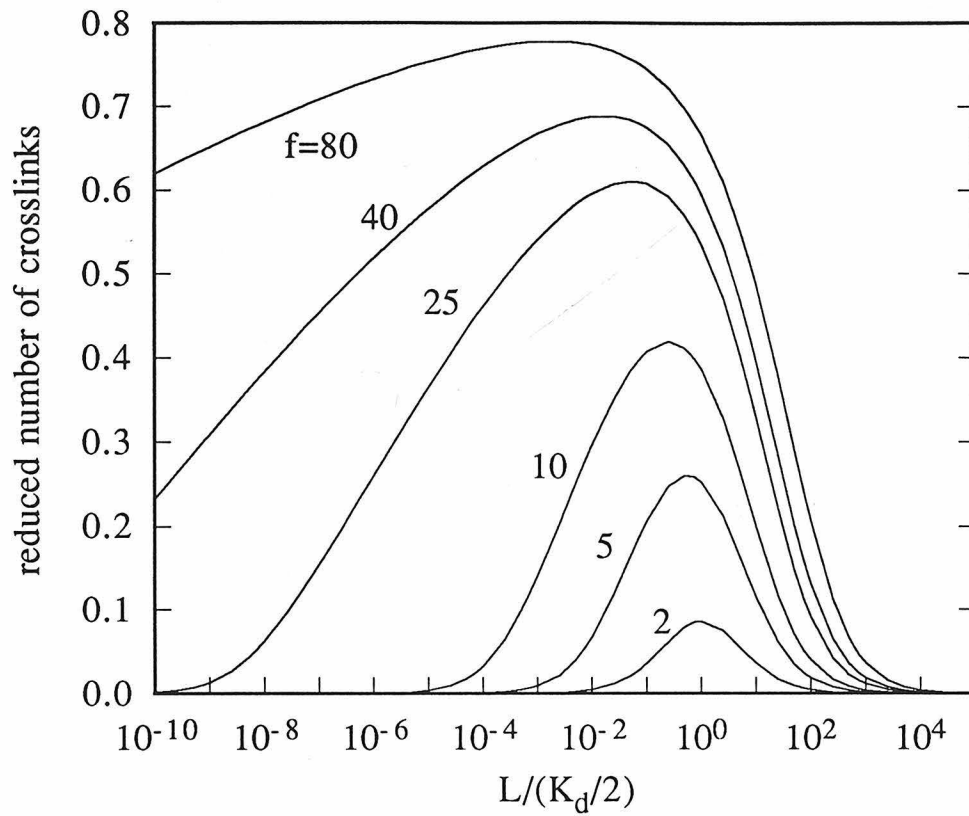


Figure 5.12. Predicted number of cross-linked receptors as a function of ligand concentration in solution. Increasing the multivalency of the ligand (f) increases the maximum cross-links, broadens the distribution of cross-links and shifts the maximum cross-links to lower ligand concentration. (From A.S. Perelson (1981) *Math. Biosci.* 53, 1-39.)

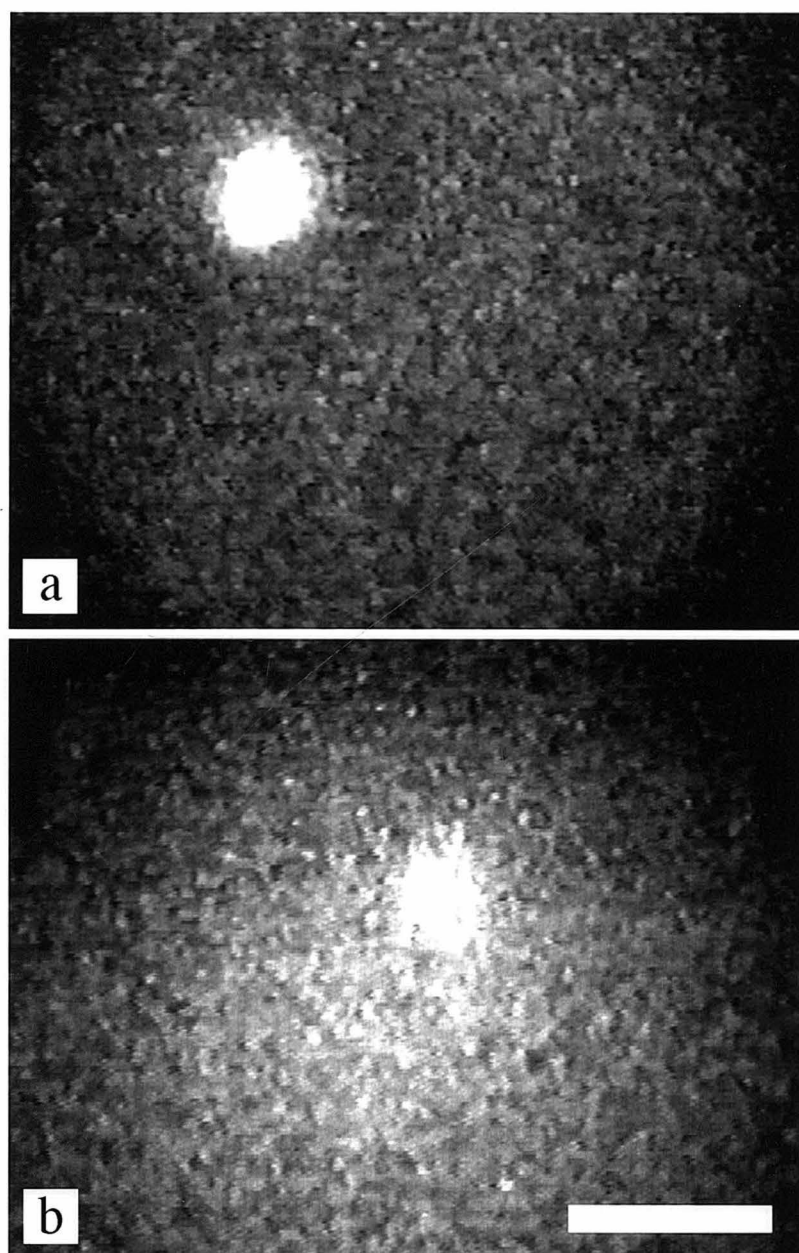


Figure 5.13. (A) Pyrene fluorescence micrograph of a 5% PSIDA-Cu/95% SOPC Langmuir monolayer in the presence of 150 nM poly(histidine) in the buffered subphase (20 mM MOPS, 100 mM NaCl, pH 7.5). (B) Rhodamine fluorescence micrograph of the same monolayer. The two bright spots in A and B, respectively, are one and the same; the monolayer has moved over the objective ca. 100 μm during the time required to change from the pyrene to the rhodamine filter set. Scale bar = 100 μm .

Appendix

This program, in Microsoft QuickC for DOS, was written to organize excimer and monomer intensities from the photon counter for determination of E/M ratios. The input file is expected to be an ASCII data file from the SR400 photon counter data acquisition software. The user defines ranges for the excimer and monomer intensities, as well as a threshold value below which counts are considered to be dark noise. Data points outside these ranges are excluded. The program calculates averages and variances for each set of excimer or monomer data points encountered. Dark counts, usually acquired when the shutter in front of the PMT is closed, are considered breaks between sets of excimer and monomer counts. The program sorts the resulting averages into four comma-separated columns, the rows of which contain an excimer average, its variance, a monomer average and its variance. The ASCII output file is suitable for importing into a spreadsheet program, such as Microsoft Excel, for determining the E/M ratios.

```
#include <c:\qc2\include\stdio.h>
#include <c:\qc2\include\math.h>
#include <c:\qc2\include\float.h>
#include <c:\qc2\include\graph.h>

#define YES 1
#define NO 0
#define DARK 0
#define EX 2
#define MON 1
#define BAD -1

main()
{

FILE *data;
FILE *res;
char file[12], result[12], yn;
int errno, go, i, j, pos;
int current, old, numcounts, numpoints;
int exsets, monsets;
long int offset = 60;
float dark, exmax, exmin, monmax, monmin, count;
```

```

float sum, mean, variance;
float exmean[400], exvar[400], monmean[400], monvar [400];
float sample[500];

/*****
/***** ENTER PARAMETERS *****/

do
{
    printf( "\n\nfilename -> " );
    scanf( "%12s", file );

    if ( (data = fopen( file, "r" )) != NULL )
    {
        go = YES;
        fflush( stdin );
    }
    else
    {
        fflush( stdin );
        go = NO;
        printf( "Error number: %d\n", errno );
        printf( "Error in opening file %12s!!!", file );
    }
} while ( go == NO );

do
{
    printf( "Write results to file -> " );
    scanf( "%12s", result );

    if ( (res = fopen( result, "w" )) != NULL )
    {
        go = YES;
        fflush( stdin );
    }
    else
    {
        go = NO;
        fflush( stdin );
        printf( "Error number: %d\n", errno );
        printf( "Error in opening file %12s!!!\n", file );
        printf( "Not a proper DOS file name?\n " );
    }
} while ( go == NO );

do
{
    dark = 0.0;
    printf( "\nDark count threshold: " );
    scanf( "%f", &dark );
    fflush( stdin );

```

```

ex:
printf( "Max excimer:  " );
scanf( "%f", &exmax);
fflush( stdin );

printf( "Min excimer:  " );
scanf( "%f", &exmin);
fflush( stdin );

if( exmin > exmax )
{
    printf( "Max excimer < Min excimer! Try again.\n\n" );
    goto ex;
}

mon:
printf( "Max monomer:  " );
scanf( "%f", &monmax);
fflush( stdin );

printf( "Min monomer:  " );
scanf( "%f", &monmin);
fflush( stdin );

if( monmin > monmax )
{
    printf( "Max monomer < Min monomer! Try again.\n\n" );
    goto mon;
}

if( (exmax>monmax && monmax>exmin) || (monmax>exmax && exmax>monmin)
)
{
    printf( "Overlapping ranges!! Try again.\n\n" );
    goto ex;
}

printf( "\n\n\n" );
printf( "Dark count threshold = %7.0f\n\n", dark );
printf( "Max excimer = %7.0f\n", exmax );
printf( "Min excimer = %7.0f\n\n", exmin );
printf( "Max monomer = %7.0f\n", monmax );
printf( "Min monomer = %7.0f\n\n", monmin );
printf( "Are these values correct? (y/n)\n");
do
    yn = tolower( getch() );
while ( yn != 'y' && yn != 'n' );
} while ( yn == 'n' );

/*****
/***** INITIALIZE VARIABLES *****/
numcounts = 0;
numpoints = 0;

```

```

sum = 0;
monsets = 1;
exsets = 1;
current = DARK;

for( j = 1; j <= 400; j += 1)
{
    exmean[j] = exvar[j] = 0.0;
    monmean[j] = monvar[j] = 0.0;
}

for( j = 1; j <= 500; j += 1 )
    sample[j] = 0.0;

/*****
/***** READ IN DATA *****/

while( fscanf( data, "%f", &count ) != EOF )
{
    /* pos = ftell( data ); */
    /* printf( "At position: %i          count = %f\n", pos, count); */
    fseek( data, offset, SEEK_CUR );

    numcounts += 1;

/*****
/***** EXCIMER, MONOMER, DARK? *****/
if( current != BAD )
    old = current;
/* printf( "%7.0f\n", count ); */

if( count <= dark )
    current = DARK;
else
    if( (count >= monmin) && (count <= monmax) )
        current = MON;
    else
        if( (count >= exmin) && (count <= exmax) )
            current = EX;
        else
        {
            current = BAD;
            /* printf( "bad point!\n" ); */
        }

/* printf( "current = %i, old = %i\n", current, old ); */

if( old == current )
{
    numpoints += 1;
    sum += count;
    sample[numpoints] = count;
}

```

```

if( current == BAD )
    /* do nothing */

if( (old != current) && ( current != BAD) )
{
    mean = sum/numpoints;                /* first finish old set */

    variance = 0.0;
    for( j = 1; j <= numpoints; j += 1 )
        variance += (sample[j] - mean)*(sample[j] - mean);
    variance = variance/(numpoints - 1);

    if( old == EX )                    /* write results of old set */
    {
        exmean[exsets] = mean;
        exvar[exsets] = variance;
    }

    if( old == MON )
    {
        monmean[monsets] = mean;
        monvar[monsets] = variance;
        printf( "%7.0f, %8.3e, %7.0f, %8.3e\n", exmean[exsets],
            exvar[exsets], monmean[monsets], monvar[monsets] );
        exsets += 1;
        monsets +=1;
    }

    if( old == DARK )

        printf( "dark count = %7.0f; variance = %8.3e\n\n", mean,
            variance);

    numpoints = 0;                    /* reset variables */
    sum = 0;
    for( j = 1; j <= 500; j += 1 )
        sample[j] = 0;

    sum += count;                    /* start new set */
    numpoints += 1;
    sample[numpoints] = count;

} /* END OF old != current LOOP */

} /* END OF WHILE != EOF LOOP */

/* once more for last data set before end of file *****/

mean = sum/numpoints;                /* first finish old set */

variance = 0.0;

```



```

for( j = 1; j <= numpoints; j += 1 )
    variance += (sample[j] - mean)*(sample[j] - mean);
variance = variance/(numpoints - 1);

if( old == EX )                                /* write results of old set */
{
    exmean[exsets] = mean;
    exvar[exsets] = variance;
}

if( old == MON )
{
    monmean[monsets] = mean;
    monvar[monsets] = variance;
    printf( "%7.0f, %6.3e, %7.0f, %6.3e\n", exmean[exsets],
            exvar[exsets], monmean[monsets], monvar[monsets] );
}

if( old == DARK )
    printf( "dark count = %7.0f; variance = %8.3e\n\n", mean,
            variance);

/* last data set written
*****/
/*****
/

printf( "\n%i data points read\n", numcounts );

/***** BAILOUT IS EARLY EXIT *****/
bailout;;

} /* END OF MAIN */

```

References

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