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CYTOCHROME C FILMS FORMED ON SILANE SELF-ASSEMBLED MONOLAYER DERIVATIZED SURFACES

by

Laurie Lynne Wood

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1998
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Laurie Lynne Wood entitled CYTOCHROME C FILMS FORMED ON SILANE SELF-ASSEMBLED MONOLAYER DERIVATIZED SURFACES and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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ABSTRACT

Formation and characterization of well-organized protein film assemblies are of high interest due to potential applications in biomolecular devices. The hypothesis that a macroscopically ordered protein film can be formed by site-directed, covalent binding of a protein to an appropriately derivatized surface formed the basis for the reported studies. The molecular architecture chosen to address this hypothesis consisted of yeast cytochrome c, a heme protein containing a unique binding site, immobilized on a surface derivatized with a silane self-assembled monolayer, (SAM). A combination of two techniques: total internal reflectance fluorescence (TIRF), to measure fluorescence anisotropy, and integrated optical waveguide-attenuated total reflectance (IOW-ATR) spectroscopy, to measure absorbance linear dichroism, was used to probe the macroscopic order of the heme groups in the film assemblies. Epifluorescence microscopy and absorbance (in an IOW-ATR geometry) were used to probe the nature of the surface-protein interactions and to determine relative protein affinities for different SAM-derivatized surfaces.

The molecular orientation distribution for yeast cytochrome c immobilized on a 100% thiol-terminated SAM was 67° ± 39°. Partial protein removal was observed under a variety of rinse conditions, indicating that multiple protein-surface interactions may have contributed to the wide distribution value. Relative binding affinity constants and protein-surface interactions are compared for yeast cytochrome c and horse heart cytochrome c on 100% thiol-capped, 100% hydroxyl-capped, and mixed SAM-modified surfaces. These studies were also extended to include a variant of yeast cytochrome c, Thr8Cys/Cys102Thr. Similar adsorption and removal trends were observed for all the protein-SAM combinations. The adsorption isotherms indicated that at least two binding processes occur during formation of each protein film, high and low affinity binding. Removal studies indicated that the adsorption process is only partially reversible. It was
concluded that employing a site-directed immobilization strategy does not necessarily produce a well-ordered protein film.
CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1. MOTIVATION AND RELEVANCE

The ability to direct the attachment of a protein to an inorganic substrate such that it is restricted to a specific orientation relative to the substrate surface is the much sought after goal of many recent research efforts [1-20]. Since the structuring of a protein film is expected to influence its functionality, an improved control over the type, extent and uniformity of protein functionality in a well-ordered film is anticipated. The creation of protein films of known orientation and functionality is important in many biotechnological fields such as biological sensor development, [21,22], biocompatibility issues (e.g., implants), [23], chromatography (e.g., affinity-based separations), [24], and nanoscale electronic devices (e.g., energy storage/conversion) [25-27]. While film formation, structure and functionality of many protein-surface combinations have been investigated under various conditions, [1-20,28-42], a clear understanding of these systems has not been established, mostly because the characterization of protein film assemblies is not trivial. Therefore, the growing interest in oriented protein film preparation has required the development of characterization techniques which are suitable not only for determining the presence of a protein film, (generally a monolayer or less), but also for probing the protein's orientation and conformation, the extent of order, and the functionality of such films. A multitude of techniques has been employed to characterize protein films, including absorbance in the UV-Visible, [9b,10,13,36b], and IR regions, [10,31,38], fluorescence, [7,13,37,44], electrochemistry, [18,29,33,34], surface plasmon resonance, [32,34], ellipsometry, [9,17,36a,37,41,42] and x-ray diffraction, [5-8,17].
For practical application of ordered protein film assemblies to such uses as biological sensors and nanoscale electronic devices, the substrate-film assembly must be stable and rugged in addition to being well-characterized, controllable and reproducible. The studies presented in this dissertation were chosen in an effort to provide essential fundamental information on systems with the potential to fit the above requirements. The main hypothesis under investigation is that a macroscopically ordered protein film could be formed by site-directed immobilization of the protein on an appropriately derivatized substrate. Other topics to be considered include the extent that non-specific binding interferes with site-directed immobilization and its effect on ordered protein film formation, as well as the effect of varying surface functionalities on protein binding - both specific and non-specific. In addition, a comparison of protein interactions with these different surfaces based on proteins of different solution thermodynamic stabilities will be investigated. The studies presented here are intended to extend and complement the current information available on protein-surface interactions and oriented protein film formation in order to produce a clearer picture of what is occurring in these systems. It is hoped that advancements in devices and components which rely on well-ordered protein films can be achieved through knowledgeable use of the results presented in this document as well as other related studies.

The basic molecular architecture investigated, (Figure 1.1), consisted of a glass substrate on which a functionalized alkyltrichlorosilane film was self-assembled. Yeast or horse heart cytochrome c was adsorbed/covalently bound to the modified surface through incubation of the derivatized substrate in a solution of the protein. This process, as well as protein removal by various rinses, was monitored by a combination of absorbance and fluorescence techniques. The molecular assembly described above and others related to it have been studied previously using various other techniques, including absorbance linear dichroism, [5c,9] and x-ray diffraction, [5,6]. The novel combination of optical techniques
Figure 1.1. Idealized Organized Protein Film Assembly on a 100% Thiol-capped SAM

A planar quartz or sol-gel waveguide substrate is modified through covalent attachment of 1-thioacetato-16-(trichlorosilyl)hexadecane. The thioacetate SAM is reduced to a thiol \textit{in situ}, followed by incubation in a solution of yeast cytochrome \textit{c} to form a disulfide-bound, ordered protein film.

Wild Type Yeast
Cytochrome \textit{c}

Thiol-capped Silane
Self-Assembled
Monolayer

Quartz or Waveguide
Substrate
presented here provides another dimension to the results reported in previous studies and has lead to further studies unique to this investigation of cytochromes c on silane SAM surfaces. In order to establish the reasoning behind the choice of each element in the assembly described above and the characterization techniques employed, background information will be presented on the methods of surface modification, immobilization techniques, protein selection and methods of characterization. In addition, various applications which require or benefit from ordered films will be described.

1.2. METHODS OF SURFACE MODIFICATION FOR MOLECULE IMMOBILIZATION

1.2.1. Introduction

There are many methods of modifying glass substrates to provide functional groups suitable for specific protein attachment, including Langmuir-Blodgett films, reconstituted membranes, polymer coatings, and self-assembled monolayers (silanes). Primarily, Langmuir-Blodgett (LB) films and self-assembled monolayers (SAMs) have been used in site-directed immobilization strategies. A brief description of these substrate derivatizations will be presented here along with references to experimental use of each system for the formation of oriented protein films.

1.2.2. Langmuir-Blodgett Films

The theory of Langmuir-Blodgett (LB) monolayer formation is described in detail by Ulman [43]. Briefly, LB films are formed by spreading a lipophilic compound, such as a fatty acid or a phospholipid, at an air-water interface and compressing the molecules with a barrier to form a liquid-crystalline film, (a Langmuir film). This film is transferred to a solid substrate by dipping the substrate through the interface either vertically (Langmuir-
LB film structure has been found to be well-ordered and controllable, including the ability to make films consisting of mixtures of lipids [43]. Thompson et al. used TIRF anisotropy to investigate the orientational order in monolayer LB films formed by transferring fluorophore-labeled lipids onto substrates as a function of surface pressure [44]. They found that the orientation distribution of a chain-labeled phospholipid did not change with surface pressure. However, the orientation distribution of a head-group labeled phospholipid does change with surface pressure, resulting in a decrease in the measured order parameter. It is suggested that the lower order parameter may be due to multiple chain tilts arising from a combination of liquid and solid domains which are formed at higher surface pressures. Therefore, choice of surface pressure for layer transfer affects the homogeneity and order of the film. Multilayers may be formed by repeated dipping, and often a bilayer is used in order to imitate a membrane surface. LeGrange et al. showed that film thickness did not appear to affect the orientation order in cadmium-stearate LB multilayers [45]. They also showed that guest molecules (in this case a fluorophore) can be homogeneously doped into a film.

Since homogeneous, well-ordered surfaces can be prepared using Langmuir-Blodgett films, in addition to providing a wide variety of surface functionalities, they have been extensively used as substrates for the formation of protein films. Both Zhao et al. [31] and Schmidt et al. [3c] used LB films doped with a biotinylated phospholipid to form avidin or streptavidin films, respectively. Pachence et al. investigated the formation and structure of cytochrome c bound electrostatically and covalently to LB film surfaces [5]. Salamon and Tollin [32] incorporated a cytochrome c oxidase into an LB film in order to study the formation of a cyt c film through complexation with the membrane-bound protein.

While the use of LB films allows a high degree of control over the surface quality and functionality, their relative fragility renders them inappropriate for application to many
device technologies. Changes in solvent conditions and temperature readily affect the integrity of these films. For example, hydrophilic films (head group out) reorient upon removal from an aqueous environment, causing collapse of a multilayer structure, and the introduction of a surfactant to the solvent disrupts the lipid or fatty acid interactions with the substrate, effectively removing the film [43].

1.2.3. Self-Assembled Monolayers

Self-assembled monolayers (SAMs) are so-called because they are formed by the spontaneous adsorption of molecules from solution (or gas phase) onto a substrate, resulting in a relatively compact, well-ordered film. One such system consists of alkylthiols which adsorb to gold substrates. The nature of the gold-sulfur interaction is not definitively known, but has been described as being somewhere between physisorption and chemisorption. Chain lengths of three to eighteen methylene units have been used to form homogeneous, densely-packed, functionalized monolayers with a chain tilt of ~ 28 - 40° from the surface normal (depending on the terminal functional group) [47]. A large selection of functional endgroups is available commercially, or can be synthesized. Since alkylthiols form SAMs on metal surfaces, they are useful for electrode modification for electrochemical analysis of protein films, as well as surface modification for use with surface analysis techniques such as: surface plasmon resonance, [34] ellipsometry, [4,24,34], x-ray photoelectron spectroscopy, [34], fluorescence microscopy, [4a], and atomic force microscopy, [34].

Knoll's group has used alkylthiol self-assembled monolayers as the first layer in the formation of supramolecular assemblies, [3d,4a], on gold substrates. These SAMs generally consist of a mixture of mercaptoundecanol and a biotinylated alkylthiol. It was found that this layer's composition affected the formation of the rest of the layers in the system [3d]. For example, the formation of an ordered layer of streptavidin bound on the
SAM depended not only on the use of an appropriate ratio of biotin-capped chains to hydroxyl-capped chains, but also on the use of a spacer on the biotin-capped chain to decrease steric hindrance from neighboring chains [4b].

Patel et al. compared immobilization of catalase protein on short alkylthiols (3-mercaptopropanoic acid) with protein immobilization on long alkylthiols (11-mercaptoundecanoic acid) as well as on mixed SAMs [34]. Either carbodiimide or N-hydroxysuccinimide (NHS) was used to link the protein to the surface. Through the use of a large variety of surface analysis techniques, it was found that the carbodiimide and NHS linkers reacted most on the mixed SAMs followed by the pure long, then pure short-chained films. The amount of protein immobilization also followed this order of preference.

Another SAM system consists of covalently bound alkylsilanes on oxide surfaces. Methoxy- or chloro-alkylsilanes are generally used for silane SAM formation. Characterization of these SAMs indicates that the most stable films are formed from trichlorosilanes, due to their ability to crosslink between chains in addition to binding to the surface at a more rapid rate than methoxy silanes. A study by Wasserman et al. determined chain orientation in homogeneous films to be nearly parallel to the surface normal, regardless of chain length [49]. The response to the need for a variety of functional group terminations on the chains and different carbon chain lengths can be found in several papers reporting syntheses of functionalized alkylchlorosilanes and in situ functionalization, [15,38,50], as well as a growing list of commercially available compounds. The ability of silanes to covalently bind on glass-type surfaces allows for modification of substrates compatible with optical spectroscopies, [9a,10,38,51], and x-ray diffraction, [6,7], as well as coating silicon substrates for ellipsometric measurements and x-ray photoelectron spectroscopy [46,52].
Lee et al. synthesized various long chain halogen-terminated trichlorosilanes and tested their ability to react with thiols [52]. The thiol reactivity of the SAMs was found to be dependent on the terminal functionality, with iodine > chlorine or bromine. A dependence on the structure of the rest of the chain was also found, where binding to the haloacetyl group was greater than to a benzyl halide, which was greater than binding to alkyl halide-terminated chains. These SAMs were used to investigate the specific binding of peptide chains through linkage to a cysteine residue.

Chain-length dependence on protein film orientation was investigated by Firestone et al., [10b]. Myoglobin, genetically modified with a unique surface cysteine residue, was covalently bound on a series of SAM surfaces in which the chain length of the precursor silane was varied. SAM characterization by IR showed that in films composed of either the long, (n>11), or short, (n<8), chains, the chains were in an all-trans configuration, which indicated an ordered film structure [10b]. SAMs of medium length chains, (n=8-11), however, showed the presence of gauche configurations, which indicated a disordered film with defect sites. The average heme tilt angle for proteins bound to the surface composed of medium length chains was close to that for random orientation. On the other hand, the average tilt angle for films formed on the short-chained SAMs indicated a non-random protein orientation. Protein films on long-chain SAMs were not considered stable due to decreases in the Soret band absorbance with time, which indicated denaturation and/or loss of the non-covalently bound heme group [10b].

The main advantage that silane SAMs provide over the other substrate modification methods is their high stability and ruggedness under a wide range of conditions. Silane SAMs have been found to be stable in pH ranges from 1-8 and at temperatures up to 120°C, as well as remaining intact under vacuum, in air, in aqueous and in many organic solutions [43,49]. Alkylthiol SAMs, by comparison, are not as stable thermodynamically nor chemically. They are desorbed at temperatures as low as 70°C and are affected by
several reagents including oxidants and halogenating agents [53]. The need for durability is of particular importance when considering applications of biosensing and nanoelectronics in which long-term stability of the substrate assembly is required under a variety of non-ideal conditions. In addition to high stability, the ease of silane film formation through incubation in a silane solution or vapor phase deposition, (which allows for coating of odd-shaped materials), makes the silane SAM ideal for many device and coating applications on an industrial level. While there is still more to be studied regarding the formation and characterization of silane SAMs, the current literature on the subject is as extensive as on most other forms of surface modification, and therefore, it can be regarded as a relatively well-characterized system.

1.2.4. Surface Modification Selection for Current Study

The choice of alkyltrichlorosilane SAMs for surface modification in these studies was two-fold. First, they covalently bind to the glass surfaces required for spectroscopic analysis, which is the primary mode of characterization selected for these protein film systems. Secondly, due to their durability, they are potentially applicable to device fabrication, and therefore the results of studies using these SAMs can be directly applied.

1.3. METHODS OF SITE-SPECIFIC PROTEIN IMMOBILIZATION

1.3.1. Introduction

Protein film formation may be directed through the use of a surface modification which attracts a unique functional site or patch on the protein surface. Site-specific immobilization requires that the protein have a unique binding site, whether it be a single amino acid residue, a group of residues which impart a specific functionality, or a surface structure that can act as a binding site. The substrate functionality is modified according to
the necessary chemistry for binding the protein through the unique site selected on the protein surface. A variety of specific interactions can be used, most of which fall into one of the following categories: physical adsorption, biospecific interactions, and covalent binding.

1.3.2. Physical Adsorption

In general, physical adsorption provides a non-specific method of protein film formation. However, under appropriate conditions, a specific region of a protein’s surface may preferentially bind to an appropriately derivatized surface. For example, a protein with an asymmetric surface charge distribution may be oriented on a negatively- or positively-charged surface through electrostatic interactions. (Figure 1.2) Electrostatic binding is the main use of physical adsorption for forming oriented films, however one can imagine the use of a hydrophobic patch or a region rich with hydrogen bonding moieties.

For example, Edmiston et al. formed site-directed physisorbed films of horse heart cytochrome c on a negatively charged arachidic acid LB bilayer [20a]. The negatively charged modified substrate was expected to attract a positive "patch" of lysine residues on the surface of cytochrome c. Orientational studies indicated a preferential ordering of the protein with respect to the surface. A high ionic strength rinse removed > 90% of the protein from the LB film, indicating that the major protein-surface interaction was electrostatic adsorption.

Chang and Herron found that acid-pretreatment of antibodies increases their surface coverage and orientation in films on hydrophobized substrates [11]. Their interpretation is that the pretreatment process conformationally affects a specific region on the antibody which is located away from the binding site (Fab region). This modified site is preferentially attracted to the surface, leaving the Fab region oriented away from the substrate and available for antigen binding.
Figure 1.2. Idealized Electrostatically Adsorbed Protein Film.

A positively charged "patch" on a protein surface is expected to be attracted to a negatively charged surface, such that preferential ordering of the protein relative to the surface is obtained.
Fraaije et al. used TIRF to investigate the effects of various adsorption conditions on the orientation of free base cytochrome c films formed on a positively or negatively charged tin oxide electrode [14].

Physical adsorption does not provide a very strong interaction for the formation of a stable, rugged protein film. The energetics of these types of interactions range from 1 to 5 kcal/mol for nonpolar interactions and up to 50-100 kcal/mol for ionic adsorption [54]. Covalent bonds, which have energies of 100-300 kcal/mol [54], are generally of more practical applicability due to their strength and specificity. Biospecific interactions, while of low to medium energy (20 kcal/mol for biotin-streptavidin, [2]), are more advantageous than physical adsorption due to their high specificity.

1.3.3. Biospecific Binding

A combination of structural and physical interactions between biological compounds may result in a high binding affinity of one species for another, generally with high selectivity. This can be seen in enzymatic reactions and antigen-antibody interactions. Therefore, it is possible to modify a substrate with one compound in order to selectively and orientationally bind its partner. One combination that has been widely studied is that of biotin binding to streptavidin (or avidin). A schematic of their structures and molecular assemblage is depicted in Figure 1.3. It was found by Blankenburg et al. [3a] and Darst et al. [3b] that oriented, 2-D crystalline domains of streptavidin were formed by binding to biotin lipid monolayers. The structure of streptavidin is such that two of its binding sites are used to attach to a biotin-modified surface, leaving two sites (on the opposite side) free to bind other biotinylated compounds.

Müller et al. [4a] and Spinke et al. [3d] used the formation of an ordered streptavidin layer as part of a multilayer system where a biotinylated Fab fragment was site-specifically bound to the streptavidin such that its active site was available to bind an
Figure 1.3. Biotin Structure and Biotin-Streptavidin Complex

Biotin with linker

\[ \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \]

\( \text{\textbullet\textbullet\textbullet} = \text{Lipid or other functional group} \)

Schematic of Biotin Complexed with Streptavidin

Biotin Symbol

\[ \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \text{\textbullet\textbullet\textbullet\textbullet} \]

Streptavidin Symbol

Biotin-Streptavidin Complex
antigen. A monoclonal antibody was then used to detect antigen binding. Overall, there were four biospecific interactions required for the formation of this system, and analyses indicated that a functional multilayer system was formed.

Through these studies and studies on other molecular assemblies, [19,28,31] it has been shown that the use of biospecific binding creates relatively stable systems which have potential use in a variety of biomolecular devices. Crystalline films produced in this manner, (such as streptavidin, [4a]), have formed ordered assemblies, and it is hoped that non-crystalline assemblies will be shown to behave in a like manner.

1.3.4. Covalent Binding

In general, covalent binding schemes used for site-specific protein immobilization utilize the functionality of a single, unique amino acid residue in the protein structure which is available for extrinsic binding, ideally without much conformational perturbation. These unique sites may be native to the protein, or introduced through genetic engineering. A tailored surface is then created to bind the protein based on the chemistry of the protein’s unique functional group. Many coupling schemes have been used, some of which are illustrated in Figure 1.4.

Bhatia et al. used heterobifunctional crosslinkers to attach antibodies to thiol terminated silane SAMs [15]. Binding occurred through lysine residues on the surface of the antibodies, of which there are many on each protein. Films of about half to one monolayer coverage were formed with very low non-specific binding. Increased antigen binding was observed for these covalently immobilized systems over previously reported values, indicating an improvement in immobilization strategy due to use of linkers.

Bohn’s group has reported the use of alkylsilanes to immobilize mutants of cyt b₃ through site-directed covalent attachment [9,51]. N-succinimidyl-6-maleimidocaproyate (EMCS) was used to couple the unique cysteine residue on the cytochrome b₃ surface to an
Figure 1.4. Common Reactions for Covalent Binding of Proteins on Substrates*

a) The cyanogen bromide technique:

b) The carbodi-imide method:

c) Via acyl groups by treatment of hydrazides with nitrous acid:

d) Coupling using cyanuric chloride:

e) Coupling through diazonium groups from aromatic amino groups

f) Coupling via thiol groups:

* Adapted from S. A. Barker in reference 21. Notation -P refers to Protein.
amino-terminated SAM. Determination of effective molar absorptivity of the surface-bound protein, surface coverage and stability in the protein films has been accomplished for these systems. The use of gas-phase silane deposition, along with the EMCS linker, improved control over the extent of protein coverage and enhanced the linker stability when compared to other covalent attachment schemes [9b]. Additional studies demonstrated control over heme orientation through the attachment of two mutants of cytochrome b₅ directly to an iodo-functionalized SAM through formation of a thioether bond. Each mutant had a unique surface cysteine residue in a different location, which led to an observed difference in the linear dichroic ratio of the heme groups in the protein film. This result was interpreted as a difference in protein orientation [9a].

1.3.5. **Choice of Immobilization Strategy**

The choice of covalent binding for the molecular architecture depicted in Figure 1.1 was based on the use of a protein, yeast cyt c, with a unique binding site (Cys102) available to form covalent bonds with thiol-reactive moieties. In addition, the use of a disulfide bonding scheme was chosen in order to have reversible binding through reduction of the disulfide bond, which would allow for confirmation of covalent bond formation. Other reasoning included the stability and control over protein orientation expected through use of covalent bonds.

1.4. **PROTEIN SELECTION FOR SITE-SPECIFIC IMMOBILIZATION**

1.4.1. **Introduction**

The choice of a protein for preparation of a model system generally involves consideration of many aspects including available information on the protein structure and functionality, information on other studies performed using that protein, and the
applicability of selected methods for analysis. In addition, a site-specific immobilization strategy requires that the protein used in film formation contain a unique site which may be used to attach it to the surface. For the systems to be investigated in this study, yeast cytochrome c (yeast cyt c) was chosen as the main protein for site-specific attachment to the thiol-capped silane self-assembled monolayers, primarily because it contains a unique cysteine residue near the surface which can form an extrinsic disulfide bond with a thiol-reactive moiety. In addition, it is relatively well-characterized, with a known crystal structure, and it contains a metal-porphyrin complex which may be probed spectroscopically. Another protein, horse heart cytochrome c (horse heart cyt c) was chosen for comparison studies due to its similarity with yeast cyt c, the major difference being the lack of the cysteine residue. A description of the properties of each protein is provided in the next section.

1.4.2. Background: Yeast and Horse Heart Cytochromes c

Both yeast and horse heart cytochromes c, (Figures 1.5 and 1.6) are readily soluble in aqueous solution, are stable over wide pH and temperature ranges, and are relatively easy to obtain in isolated forms. Therefore, they have been widely used in many studies and much is known about their structure and function [55-65]. Cytochromes c are mitochondrial electron transfer proteins which carry a single electron from a cytochrome c reductase complex to a cytochrome c oxidase complex, both membrane-bound species. Yeast and horse heart cytochromes c each consist of a single amino acid chain surrounding a single iron protoporphyrin IX prosthetic group. Yeast (Saccharomyces cerevisiae) iso-1-cytochrome c contains 108 amino acid residues and has a molecular weight of 12.5 kDa. The heme group is covalently bound to the polypeptide chain through thioether linkages at Cys14 and Cys17. Horse heart cytochrome c, 12.5 kDa, contains 106 amino acids, and also has a covalently bound heme group. The two cytochromes have very similar crystal
Figure 1.5. X-Ray Crystal Structure of Yeast Cytochrome c
Figure 1.6. X-Ray Crystal Structure of Horse Heart Cytochrome c
structures, even though their amino acid sequences are only 60% homologous [65]. The ferrocytochrome c form is about 5 kJ/mol more stable than the oxidized form.

One significant difference between the two proteins is that the yeast contains a cysteine residue at position 102, near the carboxy-terminus, which is available to bind to sulfhydryl-reactive compounds [55,61]. Another difference is that reduced horse heart cyt c is about 16 kJ/mol more thermodynamically stable than reduced yeast cyt c (the oxidized horse heart is about 11 kJ/mol more stable) [65].

Each of these proteins is suitable for absorbance studies in the UV-Visible region, having maxima at 412 nm (410 nm, horse heart), 520 nm and 550 nm in the reduced form. (Figures 1.7 and 1.8) However, the iron atom quenches fluorescence from the heme group. Removal of the iron results in a fluorescent heme moiety, but the protein’s stability is dramatically reduced. Replacement of the iron by zinc in the porphyrin produces a fluorescent analogue which is relatively stable, although less so than the iron form [33]. Spectroscopically, the planar heme group (ignoring side chains) is a circular dipole which can be studied using polarized radiation to investigate the orientation of the heme group relative to a laboratory coordinate system.

In addition to the above qualities which lend themselves well to the application of these cytochromes c (especially the yeast form) to the proposed orientation studies, yeast cytochrome c has been found to remain fairly stable and hold structural integrity upon expression with engineered variations in selected amino acids [55,66-72]. The ability to replace certain amino acid groups without significantly compromising conformation of the protein enables the use of variants for comparison to the native protein. One particular variant used in these studies was a Thr8Cys/Cys102Thr variant of iso-l-saccharomyces cerevisiae (yeast cyt c), where the threonine at position 8 (on the protein surface) was replaced with a cysteine, and the cysteine at 102 was replaced with a threonine. Essentially, this moved the thiol-reactive moiety to a position directly on the protein.
Figure 1.7. Absorbance Spectrum for Reduced Yeast Cytochrome c. Spectrum of a 180 μM protein solution in 50 mM phosphate, 100 mM NaCl, pH 6.2, in a 1 mm pathlength cuvet. Maxima are at 412, 520, and 550 nm.
Figure 1.8. Absorbance Spectrum for Reduced Horse Heart Cytochrome c. Spectrum of a 180 μM protein solution in 50 mM phosphate, 100 mM NaCl, pH 6.2, in a 1 mm pathlength cuvet. Maxima are at 410, 520, and 550 nm.
surface, which eliminates the need for a protein conformational change (slight unwinding of the carboxy terminus in the native form) in order for binding to occur. In addition to reducing the effect of binding on the protein structure, putting the cysteine at position 8 potentially alters the molecular orientation of the protein relative to the laboratory coordinate system.

1.5. METHODS OF CHARACTERIZING THIN ORGANIC AND BIOLOGICAL FILMS

1.5.1. Introduction

As stated in section 1.1, the increased interest in thin organic films, especially biological in nature, for a variety of applications, has necessitated the development or adaptation of analytical techniques for the study and characterization of such films. A review of several techniques currently being used to characterize biological thin films will be presented here. Characterization tools for analysis of self-assembled silane monolayers will be discussed in Section 2.5.

1.5.2. Electronic Absorbance Spectroscopy

Absorbance spectroscopy in the UV-VIS region has been used routinely to quantify chromophore-containing proteins. Most of these studies require transfer of the protein from the film into a solution using a large surface area (beads, or multiple planar substrates) for film formation and protein removal into a small solution volume to provide detectable solution concentrations. H.-G. Hong et al. [9b] and Lee and Saavedra [13] used solution absorbance of heme proteins removed from planar surfaces to determine surface coverages. Li and Caldwell also used solution absorbance to quantify protein desorbed from polymer-coated colloids [36b].
Absorbance due to a protein film bound to a planar substrate has been measured in a transmission geometry, [9b], however, since the absorbances are very weak, on the order of $5 \times 10^{-3}$ to $1 \times 10^{-3}$ optical density, they are often difficult to distinguish from the background. An attenuated total reflectance geometry is much more suited to absorbance measurements of thin films since the effective pathlength is increased due to multiple interactions of the incident beam with the sample. Use of integrated optical waveguides, which allow around 1000 reflections per centimeter, offer a large sensitivity increase and have been used with polarized visible radiation to assess orientation of chromophores in protein films, [9a,9c,10b,13], in addition to absorption measurements for the determination of surface coverage and adsorption isotherms [13]. Unfortunately, current available waveguide technologies only allow absorbance at a single wavelength to be monitored at a time. Therefore, no conformational information may be obtained in this manner, since an absorbance spectrum cannot be acquired.

1.5.3. Fluorescence Spectroscopy

The presence, orientation, and functionality of protein films can be assessed using fluorescence spectroscopy. Since the detection of fluorescence is inherently more sensitive than absorbance, small quantities of fluorophores, such as the amount present in a sub-monolayer protein film ($\sim 10^{-12}$ mol/cm$^2$) can be readily detected by directly probing the film. Often a total internal reflection (TIR) geometry is used to probe film fluorescence upon excitation at a single reflection. Fluorescence measurements require that the protein investigated have an intrinsic chromophore which fluoresces or that the protein be labeled with an appropriate fluorophore. Labeling a protein with a fluorophore may affect protein conformation, film formation, and/or functionality, which must be considered.

For example, Ahlers and coworkers [2] used fluorescence microscopy to quantify binding between an anti-fluorescein antibody and fluorescein-lipids doped in Langmuir
layers. They and others also used this technique to image domain formation of fluorescein-labeled streptavidin layers by binding to Langmuir films of biotin-capped lipids, among other assemblies [2,3a,4a]. Prokop et al. employed fluorescence emission of labeled Ca^{2+}-ATPase bound to amine-terminated silane SAMs to ensure film formation before further characterization by other methods [7]. Malmsten and Lassen used TIRF to compare labeled HSA (human serum albumin) films formed on hydrophobized surfaces without competition to those formed with competition from unlabeled (non-fluorescent) proteins [37]. Lee and Saavedra used polarized TIRF to determine anisotropy in cyt c and myoglobin films using fluorescent analogues of each protein [13].

1.5.4. Vibrational Spectroscopy

Infrared and Raman absorbance spectroscopies have been employed mainly to probe secondary conformational structure in protein films. Absorption bands around 1650 and 1550 cm\(^{-1}\), termed the amide I and II bands, respectively, result from a combination of C=O stretching, C-N stretching, and N-H bending vibrational modes in proteins [10a]. A particular protein conformation dictates the exact frequencies at which these absorbances occur, while shifts in these frequencies indicate conformational changes in the protein structure. For the amide I band, the spectral frequencies have been correlated to particular structures, such as α-helices, anti-parallel and parallel β-sheets and random coils both for proteins in solution and on surfaces [10a]. Several groups have employed this type of analysis to assess protein conformation and stability in films [10a,31,38]. In order to obtain sufficient signal from a protein film, FT-IR coupled with an attenuated total reflection (ATR) geometry must be employed, restricting the substrate to primarily Ge or Si. Most reported studies were done \textit{in situ} using an ATR flow cell.

Cheng et al. used ATR-FTIR to evaluate the effect of the surface functionalities of a modified substrate on the adsorption of fibronectin [38]. The ratio of β-sheet to β-turn,
based on the amide I bands, for each fibronectin-substrate combination was monitored over adsorption time to follow fibronectin conformation upon adsorption. The amide II bands were used to determine protein coverages. It was found that the different modified substrate surfaces did not affect the amount adsorbed, but did affect fibronectin conformation.

1.5.5. Surface Plasmon Resonance and Ellipsometry

Surface plasmon resonance (SPR) employs laser radiation to excite collective electronic transitions in a thin metal film on the back of a prism through total internal reflectance [32]. Absorbance of this evanescent wave of electromagnetic radiation by the substrate is modified due to the presence of a dielectric medium adsorbed to or near the metal surface. Through data analysis, the change in reflectance is related to the thickness and refractive index of an adsorbed film. Salamon and Tollin [32] used this method to obtain adsorption isotherms for cyt c on lipid membranes under varying conditions. Patel et al. [34] used SPR to show formation of catalase onto SAM-modified gold surfaces, while Spinke et al. [3d] monitored multilayer film formation of a thiol SAM-biotin-streptavidin architecture on a gold substrate.

Ellipsometry is very similar to SPR. Films are characterized by monitoring changes in the amplitude and phase of a laser beam reflected off a reflective substrate and relating these changes to the index and thickness of the film coated on the substrate. Riccio et al. used ellipsometry to confirm x-ray diffraction analysis, showing that the film thickness increased linearly with addition of lipid-protein layers [17].

1.5.6. Electrochemistry

Protein films which exhibit electron transfer or oxidation/reduction capability may be studied using electrochemical techniques. This requires film formation on an electrode,
which is generally gold, mercury, or carbon, but may also be made of tin oxide. Most electrochemical studies using mercury or carbon substrates have investigated proteins directly adsorbed on the unmodified surface. Studies involving biospecific or covalently bound protein films required surface modifications which have been done primarily using alkylthiols on gold or silanes on tin oxide.

Zhang et al. [18] used cyclic voltammetry to study the adsorption amount and conformation of cytochrome $c_3$ on mercury, glassy carbon and gold electrodes. Potential changes induced conformational changes for protein adsorbed on mercury, but not on glassy carbon nor gold. A small conformational change due to adsorption on glassy carbon was observed, but the gold surface did not appear to affect the native protein structure.

Spectroelectrochemistry was used by Collinson and Bowden [29] to determine the effect of ionic strength and electrolytes on protein adsorption. The adsorption isotherms of ferri- and ferrocytochrome $c$ adsorbed onto a tin oxide electrode were obtained by chronoabsorptometry. It was found that increases in ionic strength resulted in decreased adsorption, indicating that the electrostatic protein-surface interaction was the primary driving force for adsorption.

1.5.7. X-Ray and Electron Diffraction/Interferometry

Structured systems with some periodic order can be probed by x-ray or electron diffraction, where electron density profiles can provide information on relative film order and location relative to a substrate surface. Profile structures of multilayer protein film assemblies have been obtained in this manner by Blasie’s group at the University of Pennsylvania [5-8]. Silane SAMs or LB films on a Si-Ge multilayer substrate were used to covalently bind a monolayer of yeast cytochrome $c$ through the unique surface cysteine residue. A monolayer of photosynthetic reaction center was formed upon this film and the multilayer system was probed by x-ray diffraction techniques. A spatial resolution of 7 Å
was obtained, [6b], which allowed for each monolayer to be distinguished from the other.
While this technique can probe film thickness, and give indication of order based on the
electron density profiles, conformational structure cannot be probed due to the lack of rigid
crystallinity that would be required for such resolution. Much of the success in
determining film structures at such high relative resolution for the assembly described
above is due to the use of compounds, yeast cytochrome c and reaction center, for which
the crystal structures are known.

Diffraction techniques do not allow samples in a liquid environment to be probed,
due to interferences by bulk water, however, data may be collected under conditions of
high chamber humidity. While these techniques are powerful in elucidating film structures,
they are limited to systems with some degree of long range periodicity stemming from well-
oriented compounds.

1.5.8. Biospecific Assays

Use of a biospecific reaction to determine availability of a particular site on a bound
protein can provide information on the orientation of such compounds in a film. One
example is provided by the work of Egodage et al. [33] in which the orientation of
cytochrome c, adsorbed on a mercury electrode at specific applied potentials was probed
using two monoclonal antibodies for the adsorbed protein. ELISAs performed on each
system indicated that different denatured states were obtained depending on the adsorption
potential. Darst et al. also used a set of monoclonal antibodies to determine a nonrandom
orientation for myoglobin adsorbed on a polymer surface [16]. Comparing the affinity of
each antibody for the adsorbed protein relative to protein in solution indicated which sites
were blocked by adsorption. A nonrandom orientation was indicated by the large reduction
in the affinity of certain antibodies, while other antibody affinities decreased only slightly.
Bachas' group [19] probed the level of bioactivity of site-directed immobilized enzymes
versus randomly oriented enzymes and showed that films formed using site-directed immobilization retained a significantly greater degree of bioactivity compared to the randomly oriented films.

1.5.9. Selection of Characterization Techniques

Absorbance and fluorescence spectroscopies were chosen to characterize the proposed molecular architectures for several reasons. The selected protein contained a porphyrin heme group which was amenable to optical spectroscopic analysis. In addition, polarized absorbance and fluorescence measurements could be combined to determine the orientation distribution of the protein films in a novel set of experiments, thus providing a new method for characterizing orientation of immobilized proteins, with increased information over previous studies. The availability of waveguide technology to increase the sensitivity of absorbance measurements enabled data acquisition for the studies presented here.

1.6. APPLICATIONS FOR ORDERED PROTEIN FILMS

1.6.1. Introduction

An increasing number of technical devices are being produced using biological compounds. There are many advantages and disadvantages to the use of biological molecules for such applications as biosensors, bioelectronic devices and in bioseparations. A biomolecule, such as a protein, can be very specific in its binding capacity. It also may perform very efficient reactions which produce light/energy. However, at the same time, these molecules are very fragile, and even adsorption onto a surface for the creation of a device may affect the protein structure and its functionality. Therefore, the use of biological compounds in technical and electronic devices is a tricky procedure, requiring
control over the formation of protein assemblies and the ability to monitor the process and final product in order to ascertain that the desired outcome has been achieved. A fundamental understanding of protein-surface interactions and how functionalized surfaces affect the creation of protein films is necessary to create and optimize biotechnical devices.

The previous sections have described methods for preparing a site-directed immobilized film, including surface functionalization, choice of protein, method of attachment, and analysis techniques. This next section will provide a brief overview into some of the types of applications for which biological compounds have been used. This field is relatively new, and most of these devices have been developed over the last ten to twenty years.

1.6.2. Biosensors

The attachment of a biological compound to a transduction element for use in detecting a particular species is becoming more common [21,22]. Biosensors can be very selective due to the nature of biological compounds to react in a particular manner only with a specific analyte. Detection of that analyte relies upon signal production by the recognition event, which could be a change in absorbance, fluorescence, conformation, or electronic properties. This signal production must be unique to the binding of a specific analyte as the goal of biosensor development is to be able to use such devices in complex media, therefore reducing the need for sample cleanup.

One method of biosensor fabrication consists of protein entrapment in a polymer or sol-gel matrix which is then coated on the detection element [21]. The polymer or sol-gel matrix acts to concentrate the compounds in order to obtain sufficient signal upon analyte binding and to keep the protein from leaching out (degradation prevention). The matrix can slow analyte diffusion, however, increasing response time. In addition, it can cause steric hindrance of the entrapped molecules, decreasing the amount of recognition sites available.
By forming a layer of proteins directly on a surface, effects from a surrounding molecular matrix are avoided [21]. However, random adsorption of proteins onto a detection element may result in sterically hindered active sites. (Figure 1.9) Therefore, it is important to be able to direct the attachment of a biological compound onto a surface in an effort to optimize the detection system.

1.6.3. Bioelectronic Devices

Smaller and smaller electronic components are desired for the creation of products which take up less space and are readily portable. Oriented arrays of electroactive biological compounds can be used to create nanoscale devices which have the potential to work as efficiently and effectively as current large scale designs [25,26]. For many of these devices to work properly, the compounds must be oriented in a particular manner with respect to one another. Therefore, it is important to be able to control immobilization and orientation of film formation.

Some of the most promising devices based on biological compounds are optoelectronics consisting of optically active biological materials, such as bacteriorhodopsin, (BR). BR is a retinal-protein complex found in purple membrane, (PM), which can undergo a reversible photochemical process [26,27]. The photochemical cycle of BR has been widely applied in devices such as recording media, optical sensors, and memory cells. However, for efficient proton pumping to occur in BR-based systems, it must be uniformly oriented. A method for establishing the orientation of BR in a film of purple membrane was described by Koyama et al. [27] in which the use of bispecific antibodies resulted in orientation of the BR/PM film relative to a derivatized surface. They showed that the photoelectronic response of an oriented film was much greater than in random PM films.
Figure 1.9. Biosensor Schematic - Importance of Site-Directed Immobilization

a) Randomly adsorbed antibody showing steric hindrance to antigen binding.

b) Site-directed immobilization showing controlled orientation which reduces structural steric hindrance to antigen binding.
1.6.4. **Bioseparations**

The ability to selectively separate one compound from another is a key step in many chemical preparation and analysis procedures. While biosensors, as described in section 1.6.1, are designed to perform analyses with little to no analyte preparation (i.e. no preliminary separation requirements), this type of analysis will not be available to every sample. Affinity chromatography techniques for separation of a particular compound from a complex matrix often use immobilized antibodies or enzymes on chromatographic supports to bind an analyte, thus separating it from the solution matrix [24]. Further characterization and quantification can then be performed by continued on-column processes or removal of the analyte from the column for solution analyses. Improved column efficiency is possible by controlled immobilization of the protein to preserve its proper functionality.

1.7. **STATEMENT OF PURPOSE**

The ability to control and characterize protein films which are designed to have a specific orientation and functionality is of increasing concern for widespread application possibilities. The work presented here addresses the issues of protein-surface interactions as they relate to the formation of an oriented protein film and characterization of order in the prepared films. An attempt is made to form an ordered protein film based on selection of suitable surface derivatization, immobilization method, proteins, and characterization techniques. Although methods for site-directed immobilization of proteins have been developed and implemented, detailed structural characterization of the resulting films is not available (except for crystalline films). Current thin film analysis techniques have not been able to establish the success of site-directed immobilization strategies at producing the ordered films envisioned. The results presented here address the issue of determining the actual molecular structure obtained when a site-directed immobilization strategy is
employed. A novel combination of absorbance and fluorescence techniques is used to determine molecular orientation distributions in protein films and thin film absorbance spectrometry is used to monitor film formation and probe the nature of the protein-surface interactions. The information obtained from analysis of the protein films will add detail to the growing picture of protein film formation that is developing through the efforts of many research groups.
CHAPTER 2

FORMATION AND CHARACTERIZATION OF SELF-ASSEMBLED MONOLAYERS

2.1. INTRODUCTION

The use of silanes as coatings for modifying chromatographic supports and for increasing polymer adhesion to substrates has been well established [73]. Over the past 20 years or so, interest in silane coatings for other applications has rapidly increased, as illustrated by the large body of literature [43,49,50,52,74-83]. Part of the reason for this rise in popularity is that silanes can be covalently bonded to oxide surfaces, providing a more stable coating than those produced using adsorbed species. In addition, the process for coating substrates with silanes consists of a relatively simple solution or gas phase deposition, which has the advantages of flexibility in substrate size and shape and low labor intensity. Since the silane molecules react spontaneously with the surface during incubation to form a film, these coatings have been termed self-assembled monolayers (SAMs). In general, these silane films form a single layer with the silane group bound through oxygen to the substrate and the end group providing a new surface functionality. (Figure 2.1)

Long chain alkylsilanes (8-18 carbon residues) have been found to produce relatively well-ordered, full monolayers upon deposition [74,75,77]. Some assessments of alkylsilanes having lengths of 3-8 carbon residues indicates ordered, full-packed films of these as well [43]. Different functional groups can be put on the end of the alkylsilane chain to provide the desired surface reactivity. For the studies in this project, two different functionalities were required, one which would be reversibly sulfhydryl reactive and another which would be more hydrophilic. The two silanes chosen were a 16-carbon...
Figure 2.1. Self-Assembled Monolayer Formation (Mixed SAM Example). Deposition of silane from solvent by covalent binding to surface followed by \textit{in situ} reduction of the terminal groups.

Silane Deposition

25\% Thioacetate-/Acetate-capped

25\% Thiol-/Hydroxy-capped SAM

Quartz or Sol-Gel Waveguide Substrate
thioacetate and an 11-carbon acetate, both of which could be reduced in situ to a thiol and a hydroxyl, respectively. The thiol end group was expected to react with the thiol of the cysteine residue at position 102 in yeast cytochrome c. The hydroxyl group, being hydrophilic in nature, was to be used as a diluent for the thiol, providing a surface less conducive to protein adsorption and allowing the longer thiol chains to act as spacers for increased availability to the protein. Neither of these compounds is available commercially, however synthetic protocols have been published for the thioacetate-terminated silane [50]. Presented in this chapter are the synthetic procedures used in this study to produce thioacetate and acetate alkylsilanes, as well as characterization of the silanes and the SAMs formed by them.

2.2. SILANE SYNTHESIS

Brief outlines of the syntheses for preparing \( \text{1-}(\text{thioacetato})-16-\) (trichlorosilyl)hexadecane and \( \text{1-acetato}-11-\) (trichlorosilyl)undecane are provided here. A complete manual for the preparation of these silane compounds is provided in Appendices A and B, along with a complete list of the materials used.

2.2.1. Synthesis of \( \text{1-}(\text{thioacetato})-16-\) (trichlorosilyl)hexadecane

The synthetic route to prepare \( \text{1-}(\text{thioacetato})-16-\) (trichlorosilyl)hexadecane followed the procedure outlined by Balachander and Sukenik [50]. Briefly, \( \omega \)-undecenyl alcohol was reacted with methansulfonyl chloride, in the presence of triethylamine, to produce the mesylate. \( \omega \)-Undecenyl mesylate was then reacted with LiBr to form \( \omega \)-undecenyl bromide. A Grignard reaction was used to extend the chain length from eleven to sixteen by adding 1,5-dibromopentane. \( \omega \)-Hexadecenyl bromide was then reacted with potassium thioacetate to produce \( \omega \)-hexadecenyl thioacetate, which was then reacted with trichlorosilane in a hydrosilation reaction. The identity and purity of the final product, 1-
(thioacetato)-16-(trichlorosilyl)hexadecane, was determined by $^1$H-NMR spectrometry and the pure compound was stored in a vacuum desiccator until use.

2.2.2. Synthesis of 1-(acetato)-11-(trichlorosilyl)undecane

The synthetic route to prepare 1-acetato-11-(trichlorosilyl)undecane followed the procedure developed by Cheng [84]. $\omega$-Undecenyl alcohol was reacted with acetyl chloride in the presence of triethylamine to form $\omega$-undecenyl acetate. This compound was reacted with trichlorosilane in a hydrosilation reaction to produce 1-acetato-11-(trichlorosilyl)undecane. $^1$H-NMR spectrometry was used to identify the compound and establish purity. The silane was stored in a vacuum desiccator until use.

2.3. SUBSTRATE CLEANING

Silicon oxynitride [85] or sol-gel glass [86,119] waveguides, (fabrication of sol-gel waveguides is described in Appendix C), and quartz substrates, (Dynasil), were cleaned by rinsing with Type I water (18 MΩ-cm), scrubbing lightly with cotton soaked in 2% PCC-54 solution, (Pierce), and soaking for 20 minutes in 60°C to 80°C chromerge. After rinsing copiously with Type I water, the substrates were soaked overnight or longer in Type I water and blown dry with a stream of N$_2$ just before use.

Pieces of Si wafer (p-doped, 111 orientation, Wacker-Chemitronic GMBH) were sonicated in CHCl$_3$, rinsed in Type I water and dried with N$_2$ followed by either plasma cleaning (plasma cleaner/sterilizer, PDC-3XG, Harrick) for 10-15 minutes or soaking in room temperature chromerge overnight and rinsing in Type I water. The wafer pieces were then soaked for 2 minutes in 0.1 M KOH, then in 0.1 M HNO$_3$ for 5 minutes, followed by soaking in Type I water for 10 minutes and drying with a stream of N$_2$ just before use.

Si ATR elements were plasma cleaned for 10-15 minutes and rinsed in Type I water. They were blown dry with N$_2$ and stored in a closed container until use.
2.4. SILANE DEPOSITION AND IN-SITU REDUCTION

The deposition of 1-(thioacetato)-16-(trichlorosilyl)hexadecane and 1-acetato-11-(trichlorosilane)undecane onto quartz substrates, silicon oxynitride [85] or sol-gel glass [86,119] waveguides, (fabrication of sol-gel waveguides is described in Appendix C), or silicon wafers and subsequent in situ reduction of the functional end group to the thiol or hydroxyl, respectively, were derived from procedures described by Balachander and Sukenik, [50], and by Cheng [84].

One component silane solutions were prepared by adding a ratio of 50 µL silane to 80 mL dicyclohexyl, (0.06% (v/v) silane solution). Dicyclohexyl, (Aldrich, 99%), was used fresh from the bottle or after distillation of recycled dicyclohexyl. Mixed silane solutions were prepared by adding a total of 50 µL silane to 80 mL dicyclohexyl. Substrates, cleaned as described in section 2.3, were soaked in the silane solution at room temperature for 4 - 5 hours for thioacetate and mixed silane solutions or 1-2 hours for acetate solutions. The solutions were prepared immediately before use and containers were kept covered, but no special precautions were taken to prohibit water uptake by the solution during the reaction time. Mixed silane solutions were used only once, while single silane solutions were used 1-3 times within 48 hours of preparation in an air atmosphere of less than 20% relative humidity.

After removal of the substrates from the silane solution, they were placed in CHCl₃, (Mallinckrodt, 99.9%), and sonicated for 5 minutes, followed by sonication for 5 - 10 minutes in EtOH, (Quantum Chemical Company, Bulk, 200 Proof), and sonication for 10-15 minutes in Type I water. The substrates were then dried in a stream of N₂ and stored in a closed container under air at room temperature until use (generally overnight).

In situ reduction of the thioacetate and acetate terminal groups on the self-assembled monolayers was performed just before use of the substrate. A solution of lithium aluminum hydride, (Aldrich, 95%) in anhydrous ether, (Mallinckrodt), (approximately
0.5% (w/v)) was prepared at least 12 hours prior to use in order to allow excess LiAlH₄ to settle, leaving a saturated supernatant solution. The supernatant was pipetted off into a clean, dry container and the substrates were allowed to soak in this solution for about a minute, (or sonicated in the solution for about 30 seconds). The substrates were then rinsed in a 4% HCl, (Mallinckrodt, 37%), solution and dried in a N₂ stream. The reduction process was repeated 1-2 times. After the final time, the substrates were rinsed briefly in Type I water before drying.

Octadecyltrichlorosilane (OTS), used for some stability studies, was obtained from Aldrich and used without further purification. OTS solutions (1 % (v/v)) were prepared in dicyclohexyl. Substrates were incubated in the solution for 2 hours at room temperature, then sonicated for 5 minutes sequentially in CHCl₃, acetone, and Type I water. Silanized samples were blown dry with a stream of N₂ and stored in a closed container under air until use.

2.5. CHARACTERIZATION OF SILANE SELF-ASSEMBLED MONOLAYER SURFACES

Many surface analysis techniques have been applied to characterization of silane self-assembled monolayer (SAM) surfaces. An extensive list of surface techniques which have been applied to the characterization of SAMs along with their references is provided by Oxford and Griffin in reference 22 of their paper [80]. Most techniques probe the macroscopic nature of the SAM, such as the thickness and functionality of the monolayer. Wasserman et al. used a combination of ellipsometry, XPS and ATR-FTIR to determine that SAMs formed from C-3 to C-20 alkylsilanes of varying endgroup functionality appear to be well-packed, liquid-crystalline films [49]. Maoz and Sagiv employed wettability measurements along with quantitative and polarized ATR-FTIR to determine that well-packed monolayers are formed upon saturation of adsorption and to propose a mechanism
for the formation of silane SAMs [75]. Their polarization studies (as well as Wasserman’s studies) indicate that OTS formed films with the chains nearly perpendicular to the substrate surface, while studies by Bierbaum et al. indicate variations in chain tilt (relative to the surface normal) depending on chain length and functional end group [78]. Several groups have investigated the effect of different functional groups on wettability and functionality [46,49,81,83,87]. The SAM surfaces prepared for the experiments described in this document were characterized by five main methods: attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), contact angle measurements, ellipsometry, atomic force microscopy and x-ray photoelectron spectroscopy. A brief description of each technique is provided here along with references to more complete treatments of each subject.

2.5.1. Attenuated Total Reflectance Infrared Spectroscopy

Infrared spectra of thin film samples can be obtained with increased sensitivity using an attenuated total reflectance geometry instead of transmission. In this geometry, light is directed into an internal reflection element, IRE, at such an angle that it totally internally reflects at the IRE-air (or film) interface. At each reflection, an evanescent wave is produced which decays exponentially along the normal to the IRE surface. The intensity of the propagating light is decreased as it travels down the IRE by an absorbing species located at the IRE surface. Since the beam interacts with the surface multiple times before detection, the effective pathlength is increased. Self-assembled monolayers can be bound directly to the ATR element. In the case of silane-based SAMs, a silicon ATR element can be used to more closely approximate the SAMs bound to glass substrates.

Irrespective of the terminal functional groups, silane-derived SAMs have two regions of absorbance: the region between 950 cm⁻¹ and 1200 cm⁻¹, corresponding to Si-OH, Si-C, Si-O-Si and C-C stretches and bends, and the region between 2850 cm⁻¹ and
3000 cm\(^{-1}\), corresponding to C-H, C-H\(_2\) stretches and bends [88]. The thioacetate-terminated 
silane provides absorbance due to carbonyl stretches at 1695 cm\(^{-1}\), while the 
carbonyl stretch for the acetate absorbance is at 1742 cm\(^{-1}\) [88]. After reduction to the thiol 
and hydroxyl, respectively, these absorbance peaks disappear. The thiol terminal group is 
not active in the mid-IR, the range being monitored, so only the disappearance of the 
thioacetate peak can be used to determine that full reduction to thiol groups has been 
accomplished. The amount of terminal hydroxyl groups is small compared to the amount 
of surface hydroxyl groups and surface-bound water, so additional absorbance from O-H 
stretches in the 3300 - 3500 cm\(^{-1}\) range is difficult to determine [88]. Again, disappearance 
of the acetate is used to indicate reduction of the acetate to the hydroxyl.

Relative amounts of thioacetate to acetate functionality for mixed monolayer 
systems can be determined by ratioing their absorbances to one of the C-H peak 
absorbances. In this way, the final surface concentrations of a series of mixed monolayers 
can be determined for samples prepared using 0\% to 100\% thioacetate in solution (the 
remainder of the silane being the acetate).

The studies described here were performed using a Nicolet 510P FTIR instrument 
with an ATR attachment, (Harrick), coupled to a PC-based computer using Nicolet PC/IR 
Version 3.10 software. Si ATR crystals, (45 degree, BL Flat, 3.5 cm x 0.5 cm x 0.5 cm, 
Spectra-tech Inc.) were used as the substrate to which the silanes were bound. The 
substrates were cleaned and silanized following the procedures given in sections 2.3 and 
2.4. Mid-IR spectra (average of 300 - 500 scans) between 3300 cm\(^{-1}\) and 1500 cm\(^{-1}\) (cut-
off at lower wavenumbers due to absorbance by the Si substrate) were obtained for each 
sample.
2.5.2. Contact Angle Measurements

The wettability of a surface is dependent on its macroscopic functionality. It has been shown that by placing a solvent drop on a surface and observing the angle that this drop makes at the surface is a measure of the surface free energy (or surface tension) of a solid substrate (or a film coating a solid substrate), which can be related to its macroscopic hydrophobicity/hydrophilicity [43]. A variety of solvents have been used to evaluate solid surfaces, [43, 75, 81], and the contact angle of a particular solvent on a particular surface is indicative of the functional groups present at that surface. For example, on a very hydrophilic surface, water has a static contact angle near 0°, while on a very hydrophobic surface, the static contact angle of water is around 110° [43]. Dynamic contact angles (advancing and receding) can also be used to not only give information on surface functionality, but also provide insight into any restructuring of the molecules at the surface upon introduction and removal of the surface from a solvent [83]. One method of determining dynamic contact angles is to treat the sample like a Wilhelmy plate and relate the force on the sample upon introduction to and removal from a solvent to the contact angle of that solvent with the sample [89].

Pieces of Si wafers were used as substrates for contact angle measurements of the silane SAMs. They were cleaned and silanized according to the procedures described in sections 2.3 and 2.4. Static contact angle measurements were made by using a syringe to place a drop (approx. 20 µL) of water on the surface and taking a picture of the drop within 30 sec using a charge coupled device camera (TE/CCD-512TK/1, Princeton Instruments, Inc.) fitted with a Nikon lens and two magnifying lenses (Triffen, 52 mm, +1 and +4). Angles were determined using IPLAB software, (Spectrum, Inc.) on a Power Mac 7100/66. Dynamic contact angle data was obtained using a Cahn DCA-312 Dynamic Contact Angle Analyzer controlled by a PC computer with Cahn software. A Wilhelmy plate geometry was used, where one to two-inch square wafer samples were slowly
introduced (advancing) and withdrawn (receding) from a container of water while the force was measured as a function of distance into the water.

2.5.3. Ellipsometry

Ellipsometric measurements can be used to determine the thickness of a film on a substrate. A monochromatic, coherent wavelength of light reflected at a fixed angle from a substrate surface has a specific amplitude, \( \Psi \), and phase, \( \Delta \). [42,43]. Coating the surface with a film of a different refractive index will alter the amplitude and phase of the beam depending on the refractive index of the film and its thickness.

A Gaertner Scientific Model L116C Ellipsometer was used to determine the thickness of silane films on silica wafers, (prepared as specified in sections 2.3 and 2.4). The HeNe laser associated with the ellipsometer had a wavelength of 632.8 nm with a 45° polarization and was set at an angle of 70° relative to the sample surface. The refractive index, \( n_s \), of the silica substrate was 3.850 with an extinction coefficient, \( K_s \), of -0.020. A refractive index of 1.460 was used for both the native oxide layer and silane film [49,80]. Measurements were obtained in at least six different locations on the film for each sample.

2.5.4. Atomic Force Microscopy

A molecular-sized tip (silicon, diamond, etc.) can be attached to a cantilever and when brought close to a surface, the attractive and repulsive forces between the tip and the surface can be used to obtain a topographical “map” of the surface [43]. Information on surface roughness and macroscopic film structures can be obtained in this manner.

AFM analyses were performed by Dr. Yuezhong Du, another member of the Saavedra group, using a Nanoscope IIIa Multimode Scanning Probe Microscope (MMSPM), Digital Instruments, Inc., in tapping mode with Nanoprobe SPM TESP tips (L = 125 \( \mu \)m, \( F_o = 301-378 \) KHz). Tip-sample interactions were minimized by setting the
target amplitude at 0.5 V. The scan frequency was 1 Hz. Images were obtained on three sets of samples and each sample was probed in at least three separate locations to assure that the images obtained were real and did not contain system artifacts. In addition, the images were taken from large scale to small and back to large to insure that no tip-induced film deformations occurred. The scanning direction was always from the top to bottom of the image.

2.5.5. **X-ray Photoelectron Spectroscopy**

X-ray photoelectron spectroscopy is used to characterize surfaces by probing the binding energy of core electrons in atoms within the first few ångstroms of a surface. Core electrons are emitted from the sample surface upon excitation with x-rays. The kinetic energies of these electrons can be correlated to known values for each element, thus providing an indication of the type of atoms composing the surface.

X-ray photoelectron spectroscopy was used by Wenzler et al. [46] to observe the surface functionality of the acetate and thioacetate films and to ensure reduction to hydroxyl and thiol groups. The spectroscopy was performed using an ESCALab 2201-XL (VG Scientific, East Grinstead, U.K.). Data was collected in the following binding-energy regions: Si 2s (153.0 eV), Si 2p (103.4 eV), C 1s (284.6 eV), O 1s (531.6 eV), Cl 2p (199.9 eV), S 2s (228.0 eV), and S 2p (164.1 eV). The peak positions were assigned following the usual procedure of assigning the methylene component of the C 1s peak an energy value of 284.6 eV and linearly shifting the other peaks accordingly. High-resolution, multiplex spectra were acquired from a diameter area of ~150 µm, using a 20 eV pass energy. Fifty scans were averaged together, (~100 s per spectral region). An XPS image of the thiol SAM on an AFM tip (based on the sulfur signal) was also obtained.

XPS was used by Dr. Yuezhong Du, another member of the Saavedra group, to characterize mixed thioacetate/acetate SAM films on soda lime glass substrates. The
spectroscopy was performed using a VG\(^1\) ESCALab MarkII (VG Scientific, East Grinstead, U.K.). Data was collected and peak positions were assigned as described above. High-resolution, multiplex spectra were acquired from an area approximately 15 x 6 mm, using a 20 eV pass energy, at a 10\(^\circ\) take-off angle.

2.6. **SILANE STABILITY STUDY**

The stability of SAMs exposed to a buffer rinse was investigated using OTS SAMs on silica wafers. After SAM preparation (sections 2.3 and 2.4), half of the samples were dried in a 110°C oven for 20 minutes. A subsection of these samples was stored under air in a closed container, while others were soaked for 24 hours in 50 mM phosphate buffer, pH 7.3. Ellipsometric measurements and dynamic contact angle analyses were used to characterize the OTS SAM films as a function of drying and buffer exposure.

2.7. **RESULTS AND DISCUSSION**

2.7.1. **Silane Synthesis**

Proton NMR was used to verify the identity of the synthesized silanes and to establish relative purity. An example spectrum for 1-(thioacetato)-16-(trichlorosilyl) hexadecane is given in Figure 2.2. The set of peaks centered around 1.35 ppm are attributed to hydrogens along the alkyl chain. The peaks centered at 1.55 ppm are attributed to both the hydrogens on the carbon adjacent to the silicon atom and to the hydrogens on the carbon two bonds away from the sulfur atom. The singlet at about 2.35 ppm is due to the hydrogens from the terminal methyl group, while the triplet at 2.88 ppm is a result of the hydrogens on the carbon attached to the sulfur, (split by the hydrogens on the neighboring methylene unit). The peak at 7.25 ppm is due to the solvent, CDCl\(_3\).
Figure 2.2. $^1$H-NMR Spectrum of 1-(thioacetato)-16-(trichlorosilyl)hexadecane
Peak assignments: 1.20 - 1.65 ppm (m, 30 H), 2.32 ppm (s, 3 H), 2.88 ppm (t, 2 H), 7.25 (solvent, CDCl$_3$).
An example spectrum for 1-acetato-11-(trichlorosilane)undecane is given in Figure 2.3. This spectrum is similar to that for the thioacetate in that the hydrogens constituting the methylene chain provide a set of peaks centered at 1.35 ppm, and the peaks centered at 1.55 ppm result from the hydrogens on the carbon adjacent to the silicon atom and to the hydrogens on the carbon two bonds away from the oxygen atom. The singlet from the terminal methyl group is shifted to 2.05 ppm, and the triplet from the methylene group next to the oxygen is shifted downfield to 4.05 ppm, indicative of an acetate group.

2.7.2. ATR-FTIR Analysis of SAMs

ATR-FTIR was used to characterize the functional groups on the SAM surfaces and to verify that the reduction reactions were complete. In addition, it was used to determine the ratio of thioacetate to acetate on the surface of mixed SAMs. Example spectra of thioacetate and thiol SAMs are shown in Figures 2.4 and 2.5. Figures 2.6 and 2.7 shows example spectra of acetate and hydroxyl SAMs. A comparison of the spectra before (Figures 2.4 and 2.6) and after (Figures 2.5 and 2.7) in situ reduction of the thioacetate and acetate silanes to thiols and hydroxyls, respectively, shows that the thioacetate and acetate peaks (1695 cm⁻¹ and 1742 cm⁻¹, resp.) are no longer visible after reduction. It is possible that not all of the groups on the surface are reduced by this process, however any remaining signal is not distinguishable from the baseline.

Characterization of SAMs terminated with 100% thioacetate, 100% thiol, 100% acetate, 100% hydroxyl and mixtures of thioacetate/acetate and thiol/hydroxyl by ATR-FTIR produced the spectral data listed in Tables 2.1 and 2.2. Table 2.1 lists the peak positions for the methylene stretching frequencies for the SAMs and for crystalline and liquid alkanethiols [90]. The frequencies for the methylene stretches can be used to qualitatively estimate the crystallinity of the SAMs. Porter et al. compared the methylene stretching frequencies for a series of alkylthiols on gold, (CH₃(CH₂)ₙSH, n=3-21), with
Figure 2.3. $^1$H-NMR Spectrum of 1-(acetato)-11-(trichlorosilyl)undecane
Peak assignments: 1.20 - 1.70 ppm (m, 20 H), 2.05 ppm (s, 3 H), 4.05 ppm (t, 2 H), 7.25 ppm (solvent, CDCl$_3$).
Figure 2.4. FTIR-ATR spectrum for C-11 acetate-terminated silane SAM deposited on a silicon ATR crystal. Note: acetate carbonyl stretch at 1742 cm\(^{-1}\).
Figure 2.5. FTIR-ATR spectrum for C-11 hydroxyl-terminated silane SAM deposited on a silicon ATR crystal. Note: There is no acetate carbonyl stretch at 1742 cm⁻¹.

Nicolet Instrument Corporation

SCANS: 32 RES: 4.0 TIME: 01/11/15 28:28 FILE: C11ALCOH
Figure 2.6. FTIR-ATR spectrum for C-16 thioacetate-terminated silane SAM deposited on a silicon ATR crystal. Note: thioacetate carbonyl stretch at 1693 cm⁻¹.
Figure 2.7. FTIR-ATR spectrum for C-16 thiol-terminated silane SAM deposited on a silicon ATR crystal. Note: There is no thioacetate carbonyl stretch at 1693 cm$^{-1}$. 
Table 2.1. ATR-FTIR Methylene C-H Stretching Peak Positions for Silane SAMs and Crystalline and Liquid Alkanethiols

<table>
<thead>
<tr>
<th>Methylene C-H Stretching Modes</th>
<th>Crystalline&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Liquid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C-11 Acetate</th>
<th>C-11 Hydroxyl</th>
<th>C-16 Thioacetate</th>
<th>C-16 Thiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ν&lt;sub&gt;s&lt;/sub&gt;</td>
<td>2918</td>
<td>2924</td>
<td>2924</td>
<td>2921</td>
<td>2919</td>
<td>2920</td>
</tr>
<tr>
<td>ν&lt;sub&gt;a&lt;/sub&gt;</td>
<td>2851</td>
<td>2855</td>
<td>2853</td>
<td>2851</td>
<td>2851</td>
<td>2851</td>
</tr>
</tbody>
</table>

a) symmetric, ν<sub>s</sub>, and asymmetric, ν<sub>a</sub>.
b) Peak positions obtained by Porter et al., [90].

Table 2.2. ATR-FTIR Peak Intensities Used to Determine Mixed SAM Compositions

<table>
<thead>
<tr>
<th>Per Cent Thioacetate Silane in Solution</th>
<th>Intensity of Acetate Peak&lt;sup&gt;a&lt;/sup&gt; (1742 cm&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Intensity of Methylene Peak&lt;sup&gt;a&lt;/sup&gt; (2942 cm&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Per Cent Acetate in SAM</th>
<th>Per Cent Thioacetate in SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0380 ± 0.0034</td>
<td>0.0470 ± 0.0034</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0.0380 ± 0.0022</td>
<td>0.0499 ± 0.0025</td>
<td>94.2</td>
<td>5.8</td>
</tr>
<tr>
<td>40</td>
<td>0.0331 ± 0.0012</td>
<td>0.0542 ± 0.0024</td>
<td>75.5</td>
<td>24.5</td>
</tr>
<tr>
<td>60</td>
<td>0.0278 ± 0.0020</td>
<td>0.0566 ± 0.0013</td>
<td>60.7</td>
<td>39.3</td>
</tr>
<tr>
<td>80</td>
<td>0.0184 ± 0.0038</td>
<td>0.0628 ± 0.0046</td>
<td>32.2</td>
<td>63.8</td>
</tr>
<tr>
<td>100</td>
<td>N/A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0662 ± 0.0035</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

a) Averages of data obtained from three separate samples, (300-500 scans co-added for each sample).
b) No acetate peak present at 1742 cm<sup>-1</sup> in pure thioacetate SAM.
crystalline, \((\text{CH}_3\text{(CH}_2\text{)}_{21}\text{SH})\), and liquid, \((\text{CH}_3\text{(CH}_2\text{)}_7\text{SH})\) phase alkylthiols [90]. Their work showed an increase in crystallinity with increasing chain length. The asymmetric and symmetric methylene C-H stretches for crystalline and liquid alkane states are given in Table 2.1 along with the values for the single component acetate, hydroxyl, thioacetate and thiol SAMs. Comparison of the peak positions indicates that the C-11 acetate SAM has a liquid-like structure, while the other three SAM surfaces are fairly crystalline.

Table 2.2 contains peak intensities for the acetate carbonyl stretch and the methylene asymmetric stretch for the single-component and mixed SAMs. Relative amounts of thioacetate and acetate were based on the ratio of the peak height at 1742 cm\(^{-1}\), (acetate peak), to the methylene asymmetric stretch (2928 cm\(^{-1}\)) peak height. These ratios were normalized to the acetate/methylene ratio value for the pure acetate SAM in order to determine the relative amounts of each chain on the surface. The per cent acetate, \(A\), on the surface was therefore calculated as follows:

\[
A = \frac{I_{Ax}}{I_{Ax} + I_{Mx}} \times 100
\]

where \(I_{Ax}\) and \(I_{Mx}\) are the peak intensities from the mixed surfaces for the acetate carbonyl and the methylene peaks, respectively, and \(I_{Ao}\) and \(I_{Mo}\) are the peak intensities from the 100% acetate SAM for the acetate carbonyl and methylene peaks, respectively. These values were then converted to percent thioacetate \((100 - A)\) for discussion purposes. It was found that for a given solution mixture, a mixture of thioacetate to acetate was obtained on the surface, however, not in the same ratio. The results for comparison of the ratio of silanes in solution to the ratio of silane chains in the film are presented in Table 2.2. The low percentage of thioacetate on the surface compared to the percentage in solution was somewhat surprising since it has been observed that alkylsilanes tend to form films at
similar rates, regardless of chain length [49]. Since a covalent bonding is the primary force linking the silane chain to the surface, it is presumed that bonds irreversibly form when each trichlorosilyl group encounters the oxide surface. The deposition conditions for the films prepared in this study relied upon diffusion of the silane to the surface. Therefore, it is possible that more rapid diffusion of shorter chain silanes would bring them into contact with the surface more frequently than the long chain, thereby forming a film consisting of a relatively larger fraction of short chains.

2.7.3. Wettability of SAMs

Contact angle measurements were obtained in order to characterize the functionality of the SAM surface with respect to the degree of hydrophilicity/hydrophobicity. This information indicates the type of functional group present at the surface of the film and the macroscopic functionality imparted to the derivatized surface with respect to surface free energy [43].

a. Pure SAMs

Contact angle measurements for pure SAMs are provided in Table 2.3. The acetate surface is somewhat more hydrophilic than the thioacetate SAM. The acetate-capped chain is five methylene units shorter than that of the thioacetate and it is possible that defect sites in the acetate SAM might expose the hydrophilic glass substrate more than the thioacetate SAM. However, the value of 58° ± 3° does not compare to the literature value of 73° reported by Wasserman et al., [49]. Conditions for SAM deposition for the acetate SAM investigated by Wasserman et al. were not clearly presented, so the variation in values could be due to differing film preparations, as well as the method for measuring the contact angle.
Table 2.3. Contact Angle and Ellipsometry Measurements for Pure and Mixed SAM Surfaces formed on SiO₂ Silica Substrates

<table>
<thead>
<tr>
<th>SAM Functionality</th>
<th>Thickness (Å) (Measured)</th>
<th>Thickness (Å) (Ref.)</th>
<th>Static Contact Angle (°)</th>
<th>Reference Contact Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Acetate</td>
<td>16 ± 4</td>
<td>-</td>
<td>58 ± 3</td>
<td>73° ± 3</td>
</tr>
<tr>
<td>20% Thioacetate</td>
<td>26 ± 6</td>
<td>-</td>
<td>61 ± 3</td>
<td></td>
</tr>
<tr>
<td>40% Thioacetate</td>
<td>31 ± 3</td>
<td>-</td>
<td>65 ± 2</td>
<td></td>
</tr>
<tr>
<td>60% Thioacetate</td>
<td>23 ± 4</td>
<td>-</td>
<td>66 ± 3</td>
<td></td>
</tr>
<tr>
<td>80% Thioacetate</td>
<td>31 ± 4</td>
<td>-</td>
<td>68 ± 3</td>
<td></td>
</tr>
<tr>
<td>100% Thioacetate</td>
<td>24 ± 4</td>
<td>25-26 ± 1⁰</td>
<td>70 ± 2</td>
<td>72° ± 3/65° ± 3° a,b</td>
</tr>
<tr>
<td>100% Hydroxyl</td>
<td>16 ± 3</td>
<td>-</td>
<td>49 ± 3</td>
<td>45° ± 3</td>
</tr>
<tr>
<td>20% Thiol</td>
<td>26 ± 4</td>
<td>-</td>
<td>62 ± 1</td>
<td></td>
</tr>
<tr>
<td>40% Thiol</td>
<td>26 ± 4</td>
<td>-</td>
<td>67 ± 2</td>
<td></td>
</tr>
<tr>
<td>60% Thiol</td>
<td>23 ± 5</td>
<td>-</td>
<td>68 ± 1</td>
<td></td>
</tr>
<tr>
<td>80% Thiol</td>
<td>24 ± 4</td>
<td>-</td>
<td>70 ± 1</td>
<td></td>
</tr>
<tr>
<td>100% Thiol</td>
<td>20 ± 5</td>
<td>-</td>
<td>69 ± 1</td>
<td>71° ± 3/49° ± 3° a,b</td>
</tr>
</tbody>
</table>

a) Percentages based on silanes in solution.
b) Averages of at least 6 data points at different locations on the surface.
c) Averages of at least 3 data points at different locations on the surface.
d) Collins and Sukenik, [83].
e) Wasserman et al., [49].
f) Balachander and Sukenik, [50].
g) Advancing/Receding Contact Angle
Once the functional groups have been reduced, the 100% acetate film becomes significantly more hydrophilic, while there is no change in contact angle between the thioacetate and thiol SAMs. The hydroxyl surface contact angle of $49^\circ \pm 3^\circ$ is much closer to that reported by Wasserman et al. ($45^\circ$), [49], even though the precursor silane SAM differed (vinyl instead of acetate) as well as the method of in situ reduction (hydroboration instead of lithium aluminum hydride reduction). However, both of these values, $49^\circ$ and $45^\circ$, are much greater than contact angles reported for hydroxy 1-terminated alkylthiol SAMs (7-10°) [81]. This indicates a structural difference in the films formed with alkylsilanes versus alkylthiols. One possibility is a lower packing density for the silane SAMs, leading to exposure of the hydrophobic chain region. Another possibility is the presence of more defect sites in the silane SAMs compared to alkylthiol SAMs, which would also expose hydrophobic methylene units. Contact angles for both the thioacetate and thiol SAMs compared well with literature [50,83]. (Table 2.3) The value of $69^\circ$ could be due to the thiol group not being as hydrophilic as the hydroxyl group and/or defects in the film which reveal hydrocarbon chains.

b. Mixed SAMs

The acetate/thioacetate series of samples have contact angles which rise about $10^\circ$ over the series, (Table 2.3), as the amount of longer-chained thioacetate silanes increases. Relatively little change in contact angles is expected since the end groups of the non-reduced chains are similar to each other (-OC(O)CH$_3$ and -SC(O)CH$_3$). The slight increase in contact angle indicates an increase in hydrophobicity, which could be due to a bending over of the longer chains, exposing methylene groups as depicted in Figure 2.8. After reduction, the contact angles do not change significantly, which also supports exposure of methylene groups. It is somewhat surprising, however, that this effect would be noticed even at very low film percentages of thiol terminated chains.
Figure 2.8. Schematic of a Disordered Mixed SAM Modified Substrate
2.7.4. SAM Thickness Measurements

Ellipsometry measurements were made to determine the thickness for each of the SAM films. The thickness values can be used to verify the presence of a film and give indication of monolayer versus multilayer formation, based on comparison with literature and/or theoretical calculations.

a. Pure SAMs

The thicknesses for the pure SAMs, (Table 2.4), are lower than calculated thicknesses, which were based on estimated chain lengths, considering an all-trans configuration for the entire chain, with the chain normal to the surface [49]. The acetate or thioacetate group length was estimated using published bond lengths [91] and assuming a trans-like configuration to be 5.48 Å or 6.12 Å, respectively [49,78]. The calculated chain lengths are listed in Table 2.4. The measured thickness of 24 ± 4 Å for the thioacetate is the same as the literature value of 25 ± 1 Å, within one standard deviation, [83]. Thickness measurements for the other three SAM films have not been reported to date, so no comparison may be made. All of the values are within two standard deviations of the calculated values. The measurements are reasonable, considering that the index of the film was assumed to be 1.46 in order to elucidate a thickness value from the measurements. In addition, the film index was assumed to be a constant over the entire film area. Also, the calculated measurements to which the measured values are being compared are themselves estimates. It is possible that actual bond lengths differ and that the chains are not all-trans and/or not normal to the surface. An investigation by Whitesides et al. [49] provided indication that silane chains pack normal to the surface, and other studies have supported these results [78,81]. However chain tilts of around 14° have also been reported for silane films, which would decrease the calculated values by about 0.8 Å [43]. Such a decrease in the estimated chain length does not significantly affect the comparison to the measured values, therefore, chain tilt alone is not the source of the lower measured thicknesses.
Table 2.4. Calculated Silane Chain Lengths in Acetate/Hydroxyl and Thioacetate/Thiol SAMs

<table>
<thead>
<tr>
<th>Silane Forming SAM</th>
<th>Chain Length Normal to Surface (Å) *</th>
<th>Measured Values (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-11 Acetate</td>
<td>22</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>C-11 Hydroxyl</td>
<td>18</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>C-16 Thioacetate</td>
<td>29</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>C-16 Thiol</td>
<td>25</td>
<td>20 ± 5</td>
</tr>
</tbody>
</table>

*Based on the projection of an all-trans configuration on the surface normal as follows:

- Si-O\(^a\) 1.33 Å
- Si-C\(^a\) 1.52 Å
- Methylene\(^a\) 1.26 Å
- C-O\(^b\) 1.16 Å
- C(0)-C\(^b\) 1.24 Å
- C-S\(^b\) 1.48 Å
- C-H (methyl)\(^a\) 1.92 Å

a) Wasserman et al., [49].
b) Bond lengths from CRC Handbook of Chemistry and Physics, projected lengths calculated by trigonometry using bond angle of 109.5° [91].
b. Mixed SAMs

The thickness measurements for the mixed films varied irregularly with the increasing percentage of thioacetate silane in solution, and in general, the values were a little higher than for the pure thioacetate and thiol SAMs. One possible explanation is the formation of multilayers, especially by the acetate silane chains. The deposition time for the formation of a complete, pure acetate SAM was found to be about one hour, while formation of a complete, pure thioacetate SAM requires four to five hours, based upon AFM data collected by Dr. Yuezhong Du, another member of the Saavedra group. The AFM data is discussed in section 2.7.5. Optimal times for complete, mixed SAMs at each solution composition were not determined, rather, the deposition time used was that for the thioacetate SAM formation, (4.5 hours). Therefore, it is possible that a second layer of acetate silanes formed over the first and was not removed with washing. Theoretically, the thickness for a bilayer of acetate silanes would be around 44 Å (Table 2.4), which is thicker than the actual measured values. However, bending of chains could reduce the effective thickness. In addition, ellipsometry is a macroscopic characterization technique and the thickness measurements are averages over a relatively large area, which may include thick (multilayer) and thin (monolayer) regions.

2.7.5. AFM Characterization of SAMs

AFM images of the SAM surfaces were obtained in order to investigate the homogeneity of the derivatizing film and to determine the effect of film deposition on surface roughness. Figures 2.9 and 2.10 contain images from analysis by atomic force microscopy. The images show a fairly homogeneous surface is formed for the acetate film (Figure 2.9) after 1 hour of deposition time and the same for the thioacetate (Figure 2.10), except that the deposition time required is longer (4-5 hours). Surface roughnesses for the acetate film were on the order of 0.2 - 0.4 nm. Thioacetate films produced a slightly
Figure 2.9. Atomic Force Microscope Image of 100% Acetate SAM Deposited on a Silica Substrate (1 hour deposition)
Figure 2.10. Atomic Force Microscope Image of 100% Thioacetate SAM Deposited on a Silica Substrate (4 hour deposition)
A clean, bare silica substrate had a surface roughness of approximately 0.1 - 0.2 nm, so SAM deposition does increase the roughness of the surface slightly.

2.7.6. XPS Characterization of SAMs

Acetate and thioacetate silanes were provided to a group at the University of Utah for SAM formation and subsequent XPS analysis. Wenzler et al. performed XPS studies on pure thioacetate and acetate SAMs along with their reduced forms [46]. It should be noted that both the acetate and thioacetate silanes were of an 11-carbon chain length. The 16-carbon thioacetate silane was not provided for this set of XPS analyses. It is assumed that the results obtained for the SAM formed from the short-chained thioacetate silane adequately describe SAMs formed with the long-chained silane. This is a good assumption since the studies performed by Wenzler et al. primarily addressed the thioacetate/thiol functionalities, which would not be expected to change significantly with increasing chain length. Table 2.5 lists the major species detected for each of the films with their binding energies, in addition to their atomic concentrations measured versus their calculated molecular stoichiometry. The larger percentages of Si and O are a result of the large amount of each element present at the glass surface relative to the film. A survey scan of the acetate SAM (Figure 2.11) indicates the presence of the film through the inelastic scattering seen in the background (the rising and falling slopes). Elements buried in the layer produce more inelastic scattering than those near the film surface, such as the O 1s peak versus the C 1s peak, respectively. This trend of decreasing inelastic scattering is typical of a film assembled normal to the surface, as would be expected for these systems. (See comments in section 2.7.4a.) Information on the presence of acetate and its reduction can be obtained by looking at the C 1s peak region. A small peak is present near the main carbon peak, which indicates presence of an acetate carbon (Figure 2.12). Upon reduction,
### Table 2.5. XPS Data for Organosilane Self-Assembled Monolayers on Glass Surfaces

<table>
<thead>
<tr>
<th>Monolayer Studied</th>
<th>C 1s Chemical Shift (eV)</th>
<th>XPS Atomic %</th>
<th>Atomic % Stoich.</th>
<th>O 1s Chemical Shift (eV)</th>
<th>XPS Atomic %</th>
<th>Atomic % Stoich.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Si(CH₃)₃OC(O)CH₃</td>
<td>284.6</td>
<td>16.9</td>
<td>81.3</td>
<td>531.6</td>
<td>78.9</td>
<td>12.5</td>
</tr>
<tr>
<td>-Si(CH₃)₃OH</td>
<td>284.6</td>
<td>12.1</td>
<td>84.6</td>
<td>530.6</td>
<td>82.2</td>
<td>7.7</td>
</tr>
<tr>
<td>-Si(CH₂)₃SC(O)CH₃</td>
<td>284.6</td>
<td>8.7</td>
<td>81.3</td>
<td>531.6</td>
<td>85.9</td>
<td>6.3</td>
</tr>
<tr>
<td>-Si(CH₂)₂SH</td>
<td>284.6</td>
<td>9.7</td>
<td>84.6</td>
<td>531.6</td>
<td>84.6</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Monolayer Studied</th>
<th>Si 2p Chemical Shift (eV)</th>
<th>XPS Atomic %</th>
<th>Atomic % Stoich.</th>
<th>S 2p Chemical Shift (eV)</th>
<th>XPS Atomic %</th>
<th>Atomic % Stoich.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Si(CH₃)₃OC(O)CH₃</td>
<td>102.6</td>
<td>4.2</td>
<td>6.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-Si(CH₃)₃OH</td>
<td>101.6</td>
<td>5.7</td>
<td>7.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-Si(CH₂)₃SC(O)CH₃</td>
<td>102.6</td>
<td>5.2</td>
<td>6.3</td>
<td>164.6</td>
<td>0.2</td>
<td>6.3</td>
</tr>
<tr>
<td>-Si(CH₂)₂SH</td>
<td>102.6</td>
<td>5.3</td>
<td>7.7</td>
<td>163.6</td>
<td>0.4</td>
<td>7.7</td>
</tr>
</tbody>
</table>

a) Adapted from Wenzler et al., [46].

b) Atomic percentages calculated as described in Section 2.7.6. Only C, O, Si, and S (when present) were used in the calculation.

c) Only C, O, Si and S (when present) were used to calculate % atomic stoichiometry, based on the precursor molecule used.
Figure 2.11. XPS survey scan for C-11 acetate SAM deposited on a glass substrate. Inelastic scattering observed in the background is indicative of the layered nature of the self-assembled monolayer, [46].
Figure 2.12. High resolution XPS scan of the C 1s peak region for the acetate-terminated SAM deposited on a glass substrate. The dotted lines represent Gaussian peak fits to the data, with the acetate carbon at 288.6 eV, the ether carbon at 286.6 eV, and the methylene carbon at 284.6 eV, [46].
this peak shifts to lower energy. (Figure 2.13) For the thioacetate, a weak sulfur peak region is visible, with a peak shift to lower energy for the reduced sulfur (thiol). (Figure 2.14) An XPS image of the thiol SAM on an AFM tip (based on sulfur) shows fairly uniform coverage of the surface. [46]

XPS analysis of mixtures of thioacetate/acetate and thiol/hydroxyl SAMs was performed at the University of Arizona by Yuezhong Du, another member of the Saavedra group. The data obtained for the non-reduced and reduced mixtures are listed in Tables 2.6 and 2.7, respectively, which gives the atomic percentages for each of the major elements detected. The percentages were calculated using the following equation:

\[ C_x = \frac{I_x S_x}{\sum_i (I_i S_i)} \times 100 \]

where \( C_x \) is the atomic percentage of a given element (C, O, S or Si), \( I_x \) is the measured XPS intensity for that element (C, O, S or Si), and \( S_x \) is its sensitivity factor. The denominator is the sum of the intensity/sensitivity factor ratio for each element (C, O, S or Si). Sulfur was only included when detected.

Sulfur was only detected for the 80% and 100% (solution ratios) thioacetate and thiol SAMs. The position of the sulfur binding energy near to that for silicon makes sulfur peak detection difficult given the large amount of bulk silicon present. In addition, the 80% and 100% samples were the only two which contained over 50% thioacetate (or thiol) on the surface (Table 2.2), where the percentage is chain-related, not a percentage of sulfur to the other film components. The actual amount of sulfur relative to the rest of the film is much smaller. The limit of detection for the instrument is about 5% of the total signal, which includes not only the film, but also some of the substrate. Therefore, it is probable that the percentage of sulfur relative to the rest of the signal for the lower percentage films is below the detectable limit of the instrument.
Figure 2.13. High resolution XPS scan of the C 1s peak region for the hydroxyl-terminated SAM deposited on a glass substrate. The dotted lines represent Gaussian peak fits to the data, with the hydroxyl carbon at 286.6 eV and the methylene carbon at 284.6 eV, [46].
Figure 2.14. Overlay of high resolution XPS spectra of the S 2p region for C-11 thioacetate and thiol SAMs deposited on a glass substrate. The dotted lines represent Gaussian peak fits to the data, with the thioacetate sulfur at 163.5 eV and the thiol sulfur at 162.9 eV, [46].
Table 2.6. XPS Data for Mixed Thioacetate/Acetate Self-Assembled Monolayer Series

<table>
<thead>
<tr>
<th>Percent Thioacetate</th>
<th>C1s Chemical Shift (eV)</th>
<th>XPS atomic %</th>
<th>O1s Chemical Shift (eV)</th>
<th>XPS atomic %</th>
<th>S2p Chemical Shift (eV)</th>
<th>XPS atomic %</th>
<th>Si2p Chemical Shift (eV)</th>
<th>XPS atomic %</th>
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<tbody>
<tr>
<td>0</td>
<td>284.6</td>
<td>50</td>
<td>532.74</td>
<td>23</td>
<td>-</td>
<td>-</td>
<td>101.72, -</td>
<td>27</td>
</tr>
<tr>
<td>20</td>
<td>284.6</td>
<td>52</td>
<td>532.47</td>
<td>27</td>
<td>-</td>
<td>-</td>
<td>101.64, -</td>
<td>21</td>
</tr>
<tr>
<td>40</td>
<td>284.6</td>
<td>56</td>
<td>532.50</td>
<td>21</td>
<td>-</td>
<td>-</td>
<td>101.75, -</td>
<td>23</td>
</tr>
<tr>
<td>60</td>
<td>284.6</td>
<td>60</td>
<td>532.57</td>
<td>21</td>
<td>-</td>
<td>-</td>
<td>102.04, 98.41</td>
<td>19</td>
</tr>
<tr>
<td>80</td>
<td>284.6</td>
<td>59</td>
<td>532.52</td>
<td>20</td>
<td>163.69</td>
<td>2.3</td>
<td>102.16, 98.15</td>
<td>19</td>
</tr>
<tr>
<td>100</td>
<td>284.6</td>
<td>63</td>
<td>532.59</td>
<td>16</td>
<td>163.89</td>
<td>3.7</td>
<td>102.15, 98.11</td>
<td>17</td>
</tr>
</tbody>
</table>

a) Atomic percentages calculated as described in Section 2.7.6. Only C, O, Si, and S (when present) were used in the calculation.

b) First peak position listed is for Si2p,3/2, second peak position listed is for Si2p,1/2 (when present).

c) Atomic per cent based on combined area for both Si2p,3/2 and Si2p,1/2 peaks.
Table 2.7. XPS Data for Mixed Thiol/Hydroxyl Self-Assembled Monolayer Series

<table>
<thead>
<tr>
<th>Percent Thiol</th>
<th>C_{1s} Chemical Shift (eV)</th>
<th>XPS atomic %&lt;sup&gt;a&lt;/sup&gt;</th>
<th>O_{1s} Chemical Shift (eV)</th>
<th>XPS atomic %&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S_{2p} Chemical Shift (eV)</th>
<th>XPS atomic %&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Si_{2p} Chemical Shift (eV)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>XPS atomic %&lt;sup&gt;a,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>284.6</td>
<td>60</td>
<td>532.51</td>
<td>23</td>
<td>-</td>
<td>-</td>
<td>101.78, 97.94</td>
<td>17</td>
</tr>
<tr>
<td>20</td>
<td>284.6</td>
<td>63</td>
<td>532.49</td>
<td>19</td>
<td>-</td>
<td>-</td>
<td>101.91, 98.02</td>
<td>18</td>
</tr>
<tr>
<td>40</td>
<td>284.6</td>
<td>66</td>
<td>532.57</td>
<td>19</td>
<td>-</td>
<td>-</td>
<td>101.79, 97.98</td>
<td>15</td>
</tr>
<tr>
<td>60</td>
<td>284.6</td>
<td>68</td>
<td>532.40</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>101.80, 98.15</td>
<td>14</td>
</tr>
<tr>
<td>80</td>
<td>284.6</td>
<td>64</td>
<td>532.51</td>
<td>18</td>
<td>163.93</td>
<td>2.4</td>
<td>101.99, 98.16</td>
<td>16</td>
</tr>
<tr>
<td>100</td>
<td>284.6</td>
<td>70</td>
<td>532.42</td>
<td>16</td>
<td>163.81</td>
<td>1.8</td>
<td>101.84, 98.82</td>
<td>12</td>
</tr>
</tbody>
</table>

a) Atomic percentages calculated as described in Section 2.7.6. Only C, O, Si, and S (when present) were used in the calculation.

b) First peak position listed is for Si_{2p,3/2}, second peak position listed is for Si_{2p,1/2} (when present).

c) Atomic per cent based on combined area for both Si_{2p,3/2} and Si_{2p,1/2} peaks.
Given also in Tables 2.6 and 2.7 are the peak positions for each element (corrected for the C 1s peak). The values are close to those obtained by Wenzler et al. [46], but shifts between the non-reduced and reduced forms are not distinguishable. Again, some of these values were difficult to determine due to instrumental detection limits.

The XPS data verify the presence of SAM films with the intended functional groups and the image [46] indicates that homogeneous films are formed. The data on the pure acetate/hydroxyl and thioacetate/thiol films provide added support to the FTIR data which indicates complete (or nearly complete) reduction of the acetate groups. On the mixed SAMs, the low signal level from the sulfur leads to inconclusive results with regards to determining a correlation between the percentage of thioacetate (or thiol) on the surface and the original percentage of the silane in solution. However, there is indication of a difference between the samples, where at low percentage thiol silane in solution, the deposition does not provide enough thioacetate/thiol terminated chains to produce a signal distinguishable from background, while at the higher percentages (80-100%) a sulfur peak is visible. These results indicate that the SAM composition contains more thioacetate/thiol functional groups when there is a high percentage of their precursor chains in solution.

2.7.7. Silane Stability Study

The stability of OTS SAM films in buffer solution was investigated using contact angle measurements and ellipsometry to determine if SAM surfaces would degrade during subsequent protein deposition and protein film characterization. OTS was used as a model SAM with the assumption that its behavior is the same as the acetate/hydroxyl and thioacetate/thiol SAM films. The ellipsometry measurements and dynamic contact angle analyses for each of the sample types is summarized in Table 2.8. Included in the table are reference values for OTS SAMs on silicon wafers [80]. The thickness and contact angles for unheated SAMs are not affected by soaking in buffer.
<table>
<thead>
<tr>
<th>OTS SAM Sample</th>
<th>Thickness (Å)</th>
<th>Thickness (Å)</th>
<th>Advancing Contact Angle</th>
<th>Receding Contact Angle</th>
<th>Static Contact Angle (Literature*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Measured)</td>
<td>(Literature*)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Annealed</td>
<td>25 ± 3</td>
<td>26</td>
<td>111° ± 1°</td>
<td>96° ± 2°</td>
<td>112°</td>
</tr>
<tr>
<td>Annealed</td>
<td>24 ± 3</td>
<td></td>
<td>111° ± 1°</td>
<td>92° ± 1°</td>
<td></td>
</tr>
<tr>
<td>Non-Annealed, Buffer-Soaked</td>
<td>28 ± 3</td>
<td>-</td>
<td>111° ± 1°</td>
<td>93° ± 1°</td>
<td></td>
</tr>
<tr>
<td>Annealed, Buffer-Soaked</td>
<td>28 ± 3</td>
<td>-</td>
<td>107° ± 4°</td>
<td>87° ± 4°</td>
<td>-</td>
</tr>
</tbody>
</table>

* Ulman, [43].
It was proposed by Tripp et al. that monolayers formed using trichlorosilanes do not completely crosslink unless annealed at temperatures above 100°C [92]. Therefore, a comparison was made between heated SAMs and unheated, both with and without a buffer soak. The heat annealed SAMs appeared to be a little less stable, as indicated by the slight change in contact angle values after soaking. Two conclusions can be drawn from these data: first, heat annealing of the SAM does not improve its stability, rather it appears that stability may decrease. Secondly, based on the unheated samples, these data show that prolonged rinsing in an aqueous buffer solution does not significantly affect the SAM integrity.

2.8. CONCLUSIONS

Single component and mixed silane SAMs were characterized by ellipsometry, contact angle measurements, ATR-FTIR, XPS, and AFM. ATR-FTIR analyses indicated the presence of acetate and thioacetate functional groups and the loss of such functionalities after in situ reduction to hydroxyl and thiol groups, respectively. XPS analyses supported the ATR-FTIR results and indicated that hydroxyl and thiol terminal groups resulted from the reduction of acetate and thioacetate SAMs, respectively. AFM results revealed island growth film formation and were used to choose appropriate conditions for monolayer film formation. Contact angle measurements indicated moderately hydrophobic surfaces, with the exception of the hydroxyl-terminated SAM, which was considered hydrophilic. Ellipsometry measurements provided relative film thicknesses indicative of monolayer coverages for the one-component SAMs. Mixed SAM thicknesses were inconsistent with the expected trend, most likely a result of non-optimized deposition conditions.

The single component SAMs and the 25% thiol-terminated SAM (based on IR data) were used to modify glass-type surfaces for subsequent protein film formation. Chapters
3-5 discuss the results obtained from characterization of protein film formation and organization.
CHAPTER 3

INVESTIGATION OF YEAST CYTOCHROME C ON 100% THIOL-CAPPED SELF-ASSEMBLED MONOLAYERS

3.1. INTRODUCTION

As discussed in Chapter 1, the development of general methodologies to immobilize proteins in defined geometric orientations has been a focus of considerable research efforts in recent years [1-20]. The general approach has been to use a structurally unique site or region on the surface of the protein to geometrically "direct" the attachment of the molecule to an appropriately derivatized substrate surface. Several methods of site-directed immobilization have been investigated, such as physical adsorption via a unique functional group or chemically distinct "patch" on the surface of a protein to a chemically complementary substrate surface [7,11,12,93], biospecific adsorption, (such as the biotin-avidin complexation) [3,4,27,28, 94], and covalent bonding of a specific functional group on the surface of a protein to a pendant group on the substrate surface [6,9]. However, assessments of whether these methods actually produce oriented arrays of protein molecules are few. This shortcoming is attributable to a lack of analytical techniques appropriate for characterizing molecular orientation and macroscopic order in non-crystalline protein films at solid-liquid interfaces (i.e., films ranging from liquid crystalline to randomly oriented). Furthermore, to discriminate against artifacts, an approach that does not require the sample to be dried is preferred. Some progress has been made using polarized spectroscopic techniques [9,13,14,41,95-97], X-ray interferometry [6,7], fluorescence quenching [11], and ligand binding methods [27,98]. With some of these techniques, the mean molecular orientation of a spectral probe in a protein film can be measured. Although useful, knowledge of the mean orientation is not sufficient to assess
macroscopic order (and in order to extract a mean tilt angle, a narrow orientation distribution must be assumed). Synthetic strategies designed to produce oriented protein films could be more accurately evaluated using an analytical technique capable of also determining the distribution of molecular orientations in the film [96,99].

An experimental technique for measuring the orientation distribution of porphyrin planes in a hydrated heme protein film supported on a solid substrate is described in Appendix D. This technique was employed to examine a site-directed, covalent bonding strategy for oriented protein deposition. This is the first study of orientation distribution in a covalently immobilized protein film. Yeast cytochrome c was immobilized on thiol-capped, self-assembled monolayers (SAMs) formed from a thioacetate-terminated silane coated on planar glass substrates, (silane synthesis is described in Appendix A, SAM formation and characterization are discussed in Chapter 2). In this architecture, depicted in Figure 1.1, immobilization (presumably) occurs via disulfide bonding between the single reduced cysteine on the protein and the thiol tail group on the SAM.

3.2. EXPERIMENTAL

3.2.1. Preparation of Zinc Yeast Cytochrome c

Wild-type yeast iso-1-ferrocytochrome c from *Saccharomyces cerevisiae* (iron yeast cyt c) and Sephadex G-25 were obtained from Sigma. All other reagents were obtained from Aldrich and used without purification. Zinc yeast cyt c was prepared according to the published procedure for zinc horse heart cyt c with slight modification [85].

Apo-yeast cytochrome c was prepared from iron yeast cytochrome c by extracting the iron using HF. About 50 mg of iron yeast cytochrome c (Sigma) was transferred to a Teflon jar containing a stirbar. The jar was capped with a gas-tight lid containing inlet and outlet tubes. Nitrogen was used to purge the jar, followed by cooling with liquid nitrogen. HF was then introduced to the jar via the inlet tube, with the outlet tube closed (about 5 mL.
HF per 50 mg protein). Upon cooling, the HF condensed, forming a HF-protein solution which froze in the bottom of the jar. After thawing, the HF-protein solution was stirred in the closed container for 10 minutes. Upon further warming, the HF was allowed to evaporate; the gas bled off into a container of NaOH, leaving the dry apo-yeast cytochrome c behind in the jar. A few milliliters of 0.05 M ammonium acetate, pH 5, were used to dissolve the protein. The protein solution was purified on a Sephadex G-25 column, using ammonium acetate buffer, pH 5, as the eluent and dialyzed overnight in 0.05 M phosphate buffer containing 100 mM NaCl, pH 7.2.

Glacial acetic acid was directly added to the apoprotein solution to make a 10% (v/v) acetic acid/buffer solution. ZnCl₂ was then added in about a 10-fold molar excess and the solution was stirred for 2.5 hours in a 50°C oil bath. The zinc yeast cytochrome c solution was dialyzed against 0.05 M acetic acid, pH 3 for 7 to 8 hours, then against 0.05 M phosphate buffer with 100 mM NaCl, pH 6.2, overnight. The solution was stored in the refrigerator until use.

### 3.2.2. Protein Solution Preparation and Film Formation

Wild-type yeast iso-1-ferrocytochrome c from *Saccharomyces cerevisiae* (iron yeast cyt c) was obtained from Sigma and used without further purification. Zinc yeast cytochrome c was prepared as described in section 3.2.1. All other reagents were obtained commercially and used as received.

A 50 μM iron yeast cyt c solution was freshly prepared by weight using 50 mM phosphate buffer, pH 7.2 or pH 6.2, (for 8 hour incubation studies, the buffer also contained 100 mM NaCl, 48 hour studies did not). This solution was then diluted to 35 μM in the same buffer and its concentration verified by UV-Vis spectroscopy at 412 nm using a molar absorptivity of 96,000 M⁻¹·cm⁻¹. The concentration of the stock solution of zinc yeast cyt c was determined by UV-Vis spectroscopy using a molar absorptivity of
243,000 M$^{-1}$cm$^{-1}$ at 423 nm. (published value for zinc-substituted horse heart cyt c [64]). A diluted zinc yeast cyt c solution was prepared using a ratio of 1:8 zinc yeast cyt c:iron yeast cyt c to produce an overall yeast cytochrome c concentration of 35 μM in the phosphate buffer.

For epifluorescence and TIRF studies, quartz slides, (cleaned and silanized to form a 100% thiol-capped SAM as described in sections 2.3 and 2.4), were attached to Plexiglas fluid cells with vacuum grease. (Figure 3.1) About 1-2 mL of the 35 μM 1:8 zinc:iron yeast cytochrome c solution was placed in each cell. The slides were incubated in a closed container at room temperature for 8 or 48 hours. The cells were rinsed by pipetting off the protein solution, leaving only enough to keep the slide hydrated, and adding the phosphate buffer. The solution was swirled gently in the cell and partially removed by pipette. This process was repeated at least 6 times for each sample.

For absorbance linear dichroism, a silicon oxynitride waveguide [85] was cleaned and silanized to form a 100% thiol-capped SAM as described in sections 2.3 and 2.4. The waveguide was then placed in a flow cell (see Figure 3.2) and buffer solution was introduced to the cell. After set-up (described in section 3.2.4) and blank measurements, the flow cell was flushed with about 2 mL of 35 μM iron yeast cyt c solution and allowed to incubate for 8 or 48 hours. The cell was then flushed with 4-6 mL of buffer solution before measurements were obtained.

### 3.2.3. Epifluorescence Studies

The instrumental set-up for epifluorescence studies is shown in Figure 3.3. The source was a Nikon Model HB-1010AF Mercury lamp. A Nikon Diaphot-TMD inverted microscope was used at 10X magnification with an Omega optical filter set consisting of an excitation filter at 405 nm ± 40 nm, (Omega Optics, 405DF40), an emission filter, which
Figure 3.1. Fluid Cell for Fluorescence Studies

Plexiglass with rectangular hole

Quartz slide attached to plexiglass with vacuum grease
Figure 3.2. Flow Cell for IOW-ATR Experiments
Figure 3.3. Epifluorescence Microscopy Instrumental Set-Up

Excitation focused on surface and emission collected with 20X microscope objective

Dichroic Filter Set

Mercury Arc Lamp

CCD detection
passed light above 590 nm (Omega Optics, OG590), and a dichroic mirror which passed light above 505 nm, (Omega Optics, 505DCLPEXTO2). The emission light was routed through a monochromator (Acton Research Corp.) with a 2 mm slit width, 600 g/mm grating, and set to pass light at 590 nm. The light was detected by a photomultiplier tube, (Products for Research, Inc., Danvers, MA). Both the monochromator and PMT were controlled by a PC computer (Advance Microsystems, DMC-2304) using LabWindows software. The program used to collect data, (LabWindows, “singwvl.tst”), was written by Paul L. Edmiston. It collected and averaged five 20-second integrations with time between each integration for sample relocation.

The blank sample consisted of a silanized quartz slide hydrated with about 2 mL of buffer. Data was collected for the set of samples three times, with each data point being the average of five 20-second integrations. The following rinses were used to remove the protein film and data was collected after each rinse:

1. Low Salt Buffer Rinse (LSB) - 50 mM sodium phosphate and 100 mM NaCl, pH 7.2. Since the protein solutions for the 48 hour incubations did not contain 100 mM NaCl, it was not included in the rinses of protein films formed in this manner.

2. High Salt Buffer Rinse (HSB) - 50 mM sodium phosphate, 100 mM NaCl (for 8 hour incubations only), and 200 mM KCl, pH 7.2.

3. DTT Rinse - 50 mM sodium phosphate, 100 mM NaCl (for 8 hour incubations only), 200 mM KCl, 5 mM dithiothreitol, pH 7.2.

4. 2% Triton X-100 Rinse - 50 mM phosphate, 100 mM NaCl (for 8 hour incubations only), 200 mM KCl, 2% Triton X-100, pH 7.2.
Each cell was rinsed as described in section 3.2.2, then allowed to incubate for 30 minutes, followed by rinsing in LSB before data collection. All data was collected with LSB in contact with the surface. The data were tabulated and analyzed using a spreadsheet created in Microsoft Excel 5.0, (entitled “Epifluorescence”). The blank was subtracted from the sample fluorescence intensity and then ratioed to the blank:

\[ I_F = \frac{I_S - I_B}{I_B} \]  

where \( I_F \) is the fluorescence intensity due to the protein film, \( I_S \) is the fluorescence intensity measured for the sample, and \( I_B \) is the fluorescence intensity measured for the blank, which consisted of a quartz slide in contact with LSB. The corrected intensity values for the protein films rinsed with LSB were set to 100% fluorescence intensity and subsequent rinse intensity measurements were ratioed to those for the LSB for each sample separately.

3.2.4. Absorbance Linear Dichroism Measurements

The instrumental set-up for absorbance linear dichroism is shown in Figure 3.4. A water-cooled Innova 70 Coherent Ar Ion laser (12 W) in multiline mode at a wavelength of 514.5 nm was used as the source. The laser beam was directed by mirrors through a shutter (UniBlitz Model D122), polarizer (Newport), Fresnel half-wave rhomb (CVI FRV-2), iris, and focusing lens into the center of a rotary stage. The prism coupled waveguide in a flowcell was mounted on the rotary stage with an XYZ goniometer such that the prism corner was at the center of the stage. A NEAT-310 Programmable Stepping Motor and NEAT-620 Encoder Display, were used manually to determine the laser angle of incidence at which total internal reflection in the waveguide was achieved (mode coupling angle). (The average mode angle was determined by monitoring the intensity of the outcoupled light while rotating the stage.) A TE-cooled, Princeton Instruments, Inc. charge coupled
Figure 3.4. Diagram of IOW-ATR Set-up for Absorbance Linear Dichroism and Isotherm Data Collection

(1) Argon Ion Laser
(2) Mirror
(3) Beamsteerer
(4) Shutter
(5) Polarizer
(6) Fresnel Rhomb
(7) Iris
(8) Lens
(9) XYZ Positioner
(10) Rotary Stage
(11) Rotary Stage Stepper Motor
   Control and Encoder Display
(12) CCD Camera
(13) CCD Camera Controller
(14) Computer
device camera (TE/CCD-512TK/1) fitted with a 50 mm Nikon camera lens and two magnification lenses (Triffen, 52 mm, +1 and +4) was used to image the visible streak produced by the light traveling in the waveguide. The camera was computer controlled by a Power Mac 7100/66 using IPLab Spectrum Software.

After attaching the sample flowcell, filled with buffer, to the rotary stage and determining the mode coupling angle for TE and TM polarized light (used to determine the thickness and index of the waveguide), pictures of the streak for the blank were obtained in each polarization. The blank consisted of the assembled cell filled with 50 mM phosphate, 100 mM NaCl, pH 6.2 buffer (no NaCl was added to the buffer for the blank used with 48 hour protein incubation studies). The flowcell was then flushed using 2-3 mL of 35 μM iron yeast cyt c solution and allowed to sit for 8 or 48 hours. Before collecting pictures of the streak with the protein film, the flowcell was flushed using 4-6 mL of buffer. After all pictures had been collected, the flowcell was removed and replaced with a centimeter ruler and pictures in each polarization were obtained to determine the amount of pixels per centimeter.

Data was analyzed using IPLab Spectrum software to plot the intensity of the streak versus distance of the beam traveling down the waveguide and to measure an average background intensity. (Figure 3.5) This data was transferred to a spreadsheet in Microsoft Excel, (Loss Coefficient Worksheet), in which the data were averaged and the loss coefficient (slope) determined in each polarization. Another Excel spreadsheet, (Circular Dipole) was used to correct these values and determine the dichroic ratio as described in Appendix B.

3.2.5. Total Internal Reflectance Fluorescence Measurements

The instrumental set-up for total internal reflectance fluorescence is shown in Figure
Figure 3.5. View of Light Propagation in Waveguide as Imaged by CCD Camera and Loss Coefficient Plot Obtained Using Image

Data Analysis Results

Slope $\propto$ Absorbance

Log Intensity

Distance
3.6. The sample slide was attached via vacuum grease to a Plexiglas cell, as described in section 3.2.2. The 575 nm output from a Coherent 599 dye laser pumped by a water-cooled Innova 70 Coherent Ar Ion laser (12 W) in single line mode was used for excitation. The beam was focused with a 90 mm FL lens into a fused silica trapezoidal prism (Harrick Scientific) that was coupled to the slide with an index matching fluid. All measurements were performed at a laser power between 0.5-1 mW. The beam was a totally reflected at the surface of the slide at an incidence angle of 70°. Excitation polarization was selected using a half-wave Fresnel rhomb. Fluorescence emission was collected with a 4X objective through a bandpass filter at 635 nm ± 35 nm, (Omega Optical, 635DF35) onto a liquid nitrogen cooled CCD camera (Photometrics). The size of the image of the TIRF excitation spot on the CCD chip was typically 100 x 200 pixels. Intensity measurements were made by summing the pixel intensities over this area, using 5x5 binning and integrating for 1-5 s. The total number of pixel counts was typically 10^6-10^7. A mechanical shutter was used to block laser illumination of the sample between measurements. To negate systematic errors due to photobleaching, four measurements were made at each spot in the order: I_{TM}, I_{TE}, I_{TE}, I_{TM}, where the subscripts refer to the incident polarization. The measurements were made at five different spots spatially distributed over the surface of the slide. The mean background intensity, obtained by imaging the sample without laser illumination at each spot, was then subtracted from the mean I_{TM} and I_{TE} values. These values were subsequently corrected for polarization-dependent differences in laser power and the non-zero x-axis component of TM excitation, using the procedures outlined previously in Reference 41, to calculate I_z and I_y. These values were then corrected and used to calculate the anisotropy as described in Appendix B.
Figure 3.6. Total Internal Reflectance Fluorescence Instrumental Set-Up

TE or TM polarized laser beam

Fluorescence collected with 4X microscope objective

emission filter

PMT or CCD detection
3.2.6. Protein Surface Coverage

Surface coverages were measured for yeast cyt c immobilized on thiol SAMs using a pair of complementary absorbance and fluorescence experiments. In the absorbance assay, the absolute amount of protein removed from the substrate using a specific desorption treatment was measured. The fluorescence assay was used to determine what fraction of the total amount of adsorbed protein was removed from the substrate under identical desorption conditions. The surface coverage was then calculated from the ratio of the absolute amount desorbed (in monolayer units) to the fraction of the total amount adsorbed. Two different desorption conditions were used in both the absorbance and fluorescence assays: phosphate buffer containing 100 mM NaCl, and phosphate buffer containing 100 mM NaCl and 5 mM dithiothreitol (DTT). Thiol SAM-coated glass beads were used as the substrate for the absorbance assays due to their high surface area to volume ratio. The beads were silanized and yeast cyt c was deposited under conditions identical to those used for preparing samples for linear dichroism measurements. The amount of desorbed protein was calculated using the molar absorptivity of the native protein. Fused silica and fused quartz substrates were used for the fluorescence assays. Preparation of thiol SAMs and protein deposition were performed under conditions identical to those used for preparing samples for TIRF anisotropy measurements. Emission intensities were measured using epifluorescence microscopy as described in section 3.2.3.

3.3. RESULTS AND DISCUSSION

The primary goal of this study was to examine the hypothesis that a covalently immobilized protein film having a defined and well-ordered macroscopic orientation can be produced using site-directed bonding between a unique functional group near the surface of the protein and an appropriately derivatized substrate surface. The components of the
molecular architecture chosen to address the hypothesis are diagrammed in Figure 1.1. Yeast cyt c is immobilized on a SAM formed from a thiol-capped, hexadecyltrichlorosilane coated on a planar glass substrate. Yeast cyt c (wild-type, iso-1) was chosen because it has a single cysteine at position 102 that can form a disulfide bond with an extrinsic sulfhydryl group [55]. This cysteine therefore provides a geometrically defined site for attaching the protein via disulfide bonding to a thiol-functionalized substrate surface. This architecture is essentially identical to that used by Amador et al. [6a] and Chupa et al. [6b] in their X-ray interferometry studies of proteins immobilized to SAMs.

3.3.1. **Protein Surface Coverage**

The results of the surface coverage assays for yeast cyt c films, expressed in monolayer units, are listed in Table 3.1. These data are based on a coverage of $2.2 \times 10^{-11}$ mol/cm$^2$ as the equivalent of one monolayer, which assumes that the orientation of the close-packed molecules in the film is geometrically random and no "spreading" occurs due to adsorption-induced conformational changes [13]. Deposition from a solution containing 35 μM protein and 100 mM NaCl for a period of 8 hours produced a surface coverage of about 0.3 monolayer. In an effort to increase the coverage, the deposition was performed for 48 hours from a 35 μM protein solution that did not contain added NaCl. These differences produced an increase in coverage to approximately one monolayer. It is not known if the increased surface coverage was due to the longer deposition time, the lower ionic strength, or both.

3.3.2. **Orientation Distributions**

a. **8 Hour Protein Deposition**

The results of IOW-ATR linear dichroism and TIRF anisotropy measurements performed on immobilized films of yeast cyt c are listed in Table 3.1. The orientation
Table 3.1. Surface Coverages and Orientation Distributions for Immobilized Yeast Cytochrome c Films.

<table>
<thead>
<tr>
<th>Substrate Coating</th>
<th>Surface Coverage $^a$ (monolayers)</th>
<th>Dichroic Ratio ($\rho$)</th>
<th>Anisotropy ($r$)</th>
<th>Orientation Distribution ($\theta_\mu \pm \theta_\sigma$, deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiol SAM (8 hr deposition time)</td>
<td>0.3</td>
<td>1.27 ± 0.38 (n=3)</td>
<td>-0.163 ± 0.014 (n=3)</td>
<td>67° ± 39°</td>
</tr>
<tr>
<td>Thiol SAM (48 hr deposition time)</td>
<td>1</td>
<td>1.35 ± 0.02 (n=2)</td>
<td>-0.103 ± 0.014 (n=5)</td>
<td>n/o $^b$</td>
</tr>
</tbody>
</table>

$^a$ Based on one monolayer = 2.2 x 10$^{-11}$ mol/cm$^2$.

$^b$ Not obtainable (an orientation distribution could not be calculated due to the physical inconsistency between the measured values of $\rho$ and $r$, assuming a Gaussian distribution model).
distribution of the porphyrin planes in the film deposited on the thiol SAM for 8 hours was 67° ± 39°. From a qualitative examination of the crystal structure of yeast cyt c (Figure 1.5), the angle between the face of the protein on which cysteine 102 is located and the molecular plane of the heme appears to be in the range of 40° - 65°, depending how the cysteine side chain is "unwound" to allow disulfide bonding to the thiol SAM [30]. Thus the θ_p value of 67° for the 8 hour film is not unreasonable, assuming that the protein is immobilized as suggested in Figure 1.1.

b. 48 Hour Protein Deposition

For the yeast cyt c films formed on thiol SAMs during a 48 hour deposition period, an angular distribution could not be calculated from the pair of measured mean parameters (ρ = 1.35 and r = -0.103). In other words, these two parameters are physically inconsistent with a Gaussian distribution model for a circularly polarized oscillator with γ = 41°. However, orientation distributions could be calculated for other pairs of ρ and r that were within two standard deviations of the respective mean values, as shown in Table 3.2. The orientation distributions listed there have θ_μ values that range from 43° to 45° and θ_σ values ranging from ± 3° to ± 18°. A representative value for the orientation distribution cannot be established due to the wide range of angular distributions, since an angular distribution of ± 3° represents a highly ordered film, while ± 18° represents a moderately disordered film [31]. Even without assigning an orientation distribution to the 48 hour film, it is apparent from the difference in measured anisotropies (see Table 3.2) that the 48 hour film is structurally distinct from the 8 hour film.

c. "Site-Directed" versus "Non-Directed" Orientation Distributions

A comparison can be made between these studies of "site-directed" yeast cytochrome c films and orientation studies of horse heart cyt c adsorbed on a variety of
Table 3.2. Orientation Distributions Calculated for Selected Combinations of Emission Anisotropy and Dichroic Ratio. Application to Yeast Cyt c Films Deposited on Thiol SAMs for 48 Hours. a

<table>
<thead>
<tr>
<th>dichroic ratio (ρ)</th>
<th>Anisotropy (r)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.089</td>
<td>-0.103 ± 0.014</td>
<td>-0.117</td>
<td>-0.125</td>
</tr>
<tr>
<td>1.30</td>
<td>n/o c</td>
<td>n/o</td>
<td>43° ± 10°</td>
<td>43° ± 11°</td>
</tr>
<tr>
<td>1.32</td>
<td>n/o</td>
<td>n/o</td>
<td>n/o</td>
<td>43° ± 6°</td>
</tr>
<tr>
<td>1.35 ± 0.02 b</td>
<td>n/o</td>
<td>n/o</td>
<td>n/o</td>
<td>n/o</td>
</tr>
<tr>
<td>1.38</td>
<td>n/o</td>
<td>n/o</td>
<td>n/o</td>
<td>n/o</td>
</tr>
<tr>
<td>1.40</td>
<td>n/o</td>
<td>n/o</td>
<td>n/o</td>
<td>n/o</td>
</tr>
</tbody>
</table>

a) Gaussian orientation distributions expressed as θμ ± θσ.

b) Measured ρ and r values (mean ± standard deviation) for yeast cyt c films deposited on thiol SAMs for 48 hours.

c) Not obtainable (the combination of ρ and r did not produce a simultaneous solution to eqs 1 and 10).
surfaces [20a]. The orientation distribution values for horse heart cyt c adsorbed to an arachidic acid surface which is negatively charged and to glass derivatized with dichlorodimethylsilane (DDS, a hydrophobic surface) were found to be $46^\circ \pm 6^\circ$ and $49^\circ \pm 11^\circ$, respectively [20a]. These values indicate that fairly ordered films were formed by adsorption on these surfaces. Given the asymmetric distribution of positively charged residues over the surface of cyt c [55], it is not surprising that a film with only a moderate degree of disorder is produced when an electrostatic immobilization method is employed, as with the negatively charged arachidic acid surface. The primarily hydrophobic interaction between the protein and the DDS-treated surface is reasonable considering the hydrophobicity of a surface coated with methyl groups. The formation of an ordered film implies that one hydrophobic region on the protein surface is involved with immobilization, which is somewhat unexpected, but possible. However, it is surprising that protein films formed on thiol SAM-coated substrates are considerably more disordered, since there is only one reduced thiol group per protein, as opposed to the number of charged groups or hydrophobic regions on the cyt c surface [55]. Therefore, it would be expected that the presence of only one binding site on the protein and the formation of a covalent bond between that site and the surface would restrict the orientation of each protein forming an ordered film.

3.3.3. Protein-SAM Interactions

In the chosen system, each yeast cyt c molecule was expected to covalently bind to the thiol surface through the single cysteine residue at position 102, allowing for all of the protein molecules to have the same orientation. Thus, one would expect that if disulfide bonding was the primary interaction between the surfaces of yeast cyt c and the thiol-capped SAM, the distribution would be relatively narrow. Furthermore, if disulfide
bonding was the primary interaction, treatment with a disulfide reducing agent would cause the majority of the immobilized protein to desorb.

**a. 8 Hour Protein Deposition**

In an effort to elucidate possible causes for the surprisingly wide angular distribution of ± 39° measured for the 8 hour films, the physical nature of yeast cyt c binding to thiol SAMs was investigated. Epifluorescence microscopy of zn-yeast cyt c was used to quantitatively monitor the extent of protein desorption effected by soaking the film in buffer solutions containing a high salt concentration, DTT, or a nonionic surfactant as described in section 3.2.3. The intent was to determine what fraction of the protein film was immobilized via disulfide bonding to the thiol SAM and to find out what other interactions might be occurring between the proteins and the surface. Listed in Table 3.3 are the emission intensities measured after application of each desorption treatment, normalized to the first value measured for each film after the initial rinse in saline buffer.

The data indicate that the nature of the interaction between yeast cyt c and the thiol SAM is complex. Some fraction of the protein molecules in the 8 hour film appear to be electrostatically adsorbed, since treatment with high ionic strength buffer for 30 minutes desorbed 42% of the protein. In retrospect, this result is not surprising since it is well known that deposition of a silane monolayer on a glass surface reduces but does not eliminate its intrinsic negative charge [100]. However, it is somewhat surprising that the addition of 5 mM DTT removed very little additional protein beyond that desorbed in high ionic strength buffer. This result shows that little if any of the protein molecules are immobilized on the SAM surface *solely* through disulfide bonding. Soaking the film in high ionic strength buffer containing 2% Triton X-100 desorbed about 78% of the protein from the SAM surface. For the purposes of this discussion, the remaining 22% is
Table 3.3. Desorption of Cyt c from Thiol SAM-Coated Substrates in Salt, Dithiothreitol, and Surfactant Solutions. 

<table>
<thead>
<tr>
<th>Desorption Treatment Applied After 8 Hour Protein Deposition</th>
<th>Percentage of Initial Fluorescence Intensity Remaining After Applying Desorption Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline buffer rinse (50 mM phosphate, pH 7.2, containing 100 mM NaCl)</td>
<td>100% b</td>
</tr>
<tr>
<td>saline buffer containing 200 mM KCl; 30 min static incubation.</td>
<td>58% ± 7% (n=7)</td>
</tr>
<tr>
<td>saline buffer containing 200 mM KCl + 5 mM DTT; 30 min static incubation.</td>
<td>47% ± 13% (n=4)</td>
</tr>
<tr>
<td>saline buffer containing 200 mM KCl + 2% Triton X-100 (v/v); 30 min static incubation.</td>
<td>22% ± 4% (n=3)</td>
</tr>
</tbody>
</table>

Desorption Treatment Applied After 48 Hour Protein Deposition

<table>
<thead>
<tr>
<th>Desorption Treatment Applied After 48 Hour Protein Deposition</th>
<th>Percentage of Initial Fluorescence Intensity Remaining After Applying Desorption Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer rinse (50 mM phosphate, pH 7.2)</td>
<td>100% c</td>
</tr>
<tr>
<td>buffer containing 200 mM KCl; 30 min static incubation.</td>
<td>66% ± 11% (n=8)</td>
</tr>
<tr>
<td>buffer containing 200 mM KCl + 5 mM DTT; 30 min static incubation.</td>
<td>64% ± 9% (n=4)</td>
</tr>
<tr>
<td>buffer containing 200 mM KCl + 2% Triton X-100 (v/v); 30 min static incubation.</td>
<td>52% ± 10% (n=4)</td>
</tr>
</tbody>
</table>

a) Films were formed by adsorption from solutions containing 1:8 Zn-cyt c:cyt c, under the same conditions used to form films for orientation distribution measurements (see text). Epifluorescence emission intensities measured after application of each desorption treatment were normalized to the first value measured for each film after the initial rinse in phosphate buffer.

b) Surface coverage of ca. 0.3 monolayer.

c) Surface coverage of ca. 1.0 monolayer.
considered "irreversibly" adsorbed. This result strongly implicates hydrophobic interactions as a major contributor to the forces that immobilize yeast cyt c to thiol SAMs. For each of the desorption treatments, extending the soaking period to 24 hours did not result in additional protein removal beyond that observed at 30 min.

b. 48 Hour Protein Deposition

Desorption experiments were also performed on yeast cyt c films formed by 48 hour deposition on thiol SAMs. The experimental procedure was identical to that described above, except that the buffer rinse did not contain 100 mM NaCl (since the protein deposition solution also did not contain added NaCl). The results, listed in Table 3.3, were similar to those observed for the 8 hour films. The only significant difference is that 50% of the protein film was removed by soaking in the 2% surfactant solution, leaving 50% "irreversibly" adsorbed. This change is not unreasonable considering the increased incubation time during which some protein unfolding or reordering could occur, resulting in multiple adsorptive interactions with the surface.

c. "Site-Directed" versus "Non-Directed" Films

The nature of the protein interactions with the surfaces for adsorbed films of horse heart cyt c was probed [20a]. In both cases where a narrow orientation distribution was present, a single type of noncovalent interaction (electrostatic or hydrophobic, respectively) appeared to dominate the adsorptive interaction between the surfaces of the protein and the substrate. By comparison, relative disorder, ($\theta_\alpha \geq \pm 20^\circ$), was measured for films of horse heart cyt c adsorbed on three other types of substrates [20a]. For these systems, there was no single type of protein-surface interaction which dominated adsorption. Rather, a combination of multiple forces acted to immobilize the protein on the surface.
From these results, there appears to be a relationship between the degree of order in a film and the number of different interactions present between the protein and the surface.

### 3.3.4. The Molecular Picture

The lack of protein desorption observed when protein films were incubated with DTT raises the question of chemical availability of the thiol tail groups on the SAM. This issue was assessed by reacting thiol SAM-coated substrates with SAMSA fluorescein (SF), Figure 3.7, which forms disulfide bonds with reduced thiols [101]. SAMs formed using octadecyltrichlorosilane (OTS) were used as a control to assess the extent of nonspecific adsorption, and epifluorescence microscopy was employed to monitor SF adsorption and desorption from SAMs. The extent of SF binding to thiol SAMs was 15-fold greater than on OTS SAMs, indicating that specific chemisorption predominated over nonspecific physisorption. Soaking SF-treated, thiol SAMs in buffer containing 2 mM DTT for 1 hour caused 50% of the surface-bound SF to desorb. From these results, we conclude that a significant fraction of the SF adsorbed on thiol SAMs was immobilized via disulfide bonding. Although these experiments do not provide information on the percentage of reactive thiol groups on the SAM, they do demonstrate that at least some of the thiols are accessible to a sulfhydryl-reactive molecule. In this context, it is important to note that based on the projected areas (onto the x-y plane) of a C\textsubscript{16}SH monomer and cyt c, less than 5% of the tail groups on the thiol SAM must be accessible to enable a close packed monolayer of yeast cyt c to be chemisorbed via disulfide bonding.

It is clear from the data listed in Table 3.3 that several types of interactions exist between the surfaces of yeast cyt c and thiol SAM-coated glass. In other words, a substantial fraction of the immobilized protein molecules are nonspecifically adsorbed in both the 8 hour and 48 hour films. (Here the term "nonspecific" is used to refer to any protein-SAM interaction other than pure disulfide bonding at cysteine 102). This result is
**Figure 3.7.** Structure of SAMSA Fluorescein

**SAMSA Fluorescein**

5-((2-(and-3)-S-(acetylmercapto)succinoyl)-amino)fluorescein

MW = 521  
Excitation $\lambda = 495$ nm  
Emission $\lambda = 520$ nm

(-H, -SC(O)CH$_3$)
not surprising given the well-known tendency of many proteins to adsorb to virtually any surface [61b]. It is possible that the addition of DTT did reduce disulfide bonds formed between the Cys 102 and the thiol SAM. However, since the DTT treatment did not cause significant desorption, the protein must be immobilized by forces other than (or in addition to) disulfide bonding. The substantial degree of nonspecific adsorption is one probable cause of the relatively broad angular distribution of ±39° measured for the 8 hour film. In this scenario, the protein interacts with the thiol SAM surface in a variety of geometric orientations, generating a broader orientation distribution than expected based on the idealized geometry depicted in Figure 1.1.

Conformational change is a second possible cause for the broad orientation distribution in the 8 hour film. In this scenario, a substantial fraction of the immobilized protein molecules undergo adsorption-induced conformational changes [102]. If these changes perturb the geometric relationship between the heme plane and polypeptide matrix that surrounds it, and if the degree of perturbation varies among the molecules, a broad molecular orientation distribution would result. Furthermore, the occurrence of a conformational change would probably lead to formation of additional, nonspecific adsorptive contacts between the protein and the substrate [102]. It is unlikely that reduction with DTT would be sufficient to quantitatively desorb molecules that were both specifically and nonspecifically bound. In a recent paper, Tobias et. al [103] used molecular dynamics simulations to study the conformation of a yeast cyt c molecule disulfide bonded to a thiol SAM. Their results predict that the protein undergoes minor structural changes when it partially "dissolves" into the SAM (i.e., the polar side chains of the protein extend to "wet" the SAM surface). Thus, tethering the protein to the SAM is predicted to result in the formation of additional, noncovalent binding interactions.

To date there is no experimental data that describes the conformational state of cyt c immobilized on thiol SAMs. Therefore, at this point it cannot be determined if the
orientational disorder in the 8 hour film is due to:  

i) a substantial degree of nonspecific protein adsorption, which produces a broad distribution of geometric orientations; 

ii) a considerable variation in the extent of conformational change among adsorbed protein molecules, which also results in a broad distribution of geometric orientations; or 

iii) a combination of i and ii.

3.4. CONCLUSIONS

Developing an experimental approach designed to immobilize a macroscopically oriented protein film at a solid-liquid interface consists of two major steps: 1) devising an experimental strategy and using it to construct the molecular assembly; and 2) evaluating if the experimental strategy successfully produced the molecular assembly that was envisioned. Numerous studies have addressed the first step [4-17]. However, due to the difficulty of measuring molecular orientation in hydrated protein films, few groups have attempted to address the second step. The results reported here are the first direct measurements of the molecular orientation distribution in a hydrated protein film immobilized using a site-directed bonding strategy.

Overall, the results show that the molecular architecture selected for this initial study, yeast iso-1-cyt c disulfide bonded to a thiol-capped SAM, does not produce a highly oriented film. A significant fraction of the immobilized protein molecules nonspecifically interact with the SAM, which probably contributes to the relatively broad orientation distribution of heme groups in the film. The extent to which nonspecific protein-substrate interactions compete with the desired, specific interaction is certainly a key issue for production of protein films where orientation is an important consideration. This molecular architecture was chosen based on previous studies [6] which indicated that the protein formed an ordered film on this type of surface. Amador et al. [6a] and Chupa et al. [6b] used X-ray interferometry to investigate the orientation of yeast cyt c attached to an 11-
siloxyundecanethiol SAM. It is to be noted that this silane chain was shorter than that used in the studies presented here (16-siloxyhexadecanethiol SAM) and that both surface modification conditions and protein film formation conditions differed from those used in the studies which are presented in this document. Prolonged soaking of the protein coated substrate in a DTT solution (20 hours) was shown to remove all of the protein film based on transmission absorbance spectroscopy. The x-ray interferometry was able to provide information on the location of electron density with respect to the surface. The position of this density indicates that the proteins are oriented with the long axis parallel to the substrate surface. (7Å resolution), [6]. The results presented in this chapter do not support the molecular picture presented in the other studies [6] and imply that to definitively assess the utility of a methodology designed to produce an oriented protein film at a solid-liquid interface, the use of an experimental approach capable of characterizing the molecular orientation distribution in the film is required.
CHAPTER 4

COMPARISON OF YEAST CYTOCHROME C AND HORSE HEART
CYTOCHROME C INTERACTIONS ON 100% THIOL-CAPPED SAMS
WITH 100% HYDROXYL-CAPPED SAMS AND MIXED SAMS

4.1. INTRODUCTION

From the results in Chapter 3, it is apparent that the system as depicted in Figure 1.1 did not satisfy the hypothesis that a well-ordered protein film could be formed through site-directed covalent attachment to an appropriately modified surface. A key phrase in this statement is “appropriately modified surface”. The results of the removal studies in Chapter 3 and the other studies to which they are compared suggest that in order to have an oriented protein film, protein-surface interactions need to be reduced to one major (>90%) interaction. Therefore, in order to alter the system in an attempt to produce a well-ordered film, a different surface functionalization was chosen, still using alkylsilane SAMs. The idea behind varying the surface functionality was to augment the extent of the desired attachment, that being the formation of a disulfide bond between a thiol and the Cys102 on the protein, while reducing the amount of non-specifically bound protein. Since the Cys102 moiety is in a small pocket and not directly on the protein surface, some unfolding of the protein is necessary for binding to occur. The 100% thiol film presented a relatively flat surface (at least theoretically), in which all of the thiols were of equal height. This may have made the approach of the protein to the surface difficult for binding to occur without other interactions (electrostatic, etc.) between the surface and other residues on the protein.

It was shown in studies by Knoll’s group that the binding of streptavidin to a surface comprised of a mixture of mercaptoundecanol and a biotinylated alkylthiol on gold was increased upon the use of a spacer on the biotin-capped chain to decrease steric...
hindrance from neighboring chains [4b]. Therefore, a diluent for the thioacetate silane was chosen with a shorter chain length to produce a “tether effect”, where the thiol-capped chains would stick up above the diluent chains providing easier access for the protein to form a disulfide bond.

In order to make the surface less attractive to non-specific adsorption, the diluent functionality was chosen to imitate a more aqueous environment, so an acetate-capped silane which could be reduced to a hydroxyl group \textit{in situ} was employed. The mode of reduction was the same as that for the thioacetate group such that a combination of thioacetate and acetate groups could be reduced simultaneously to form a thiol/hydroxyl surface. The percentage of thiol-capped film was chosen somewhat at random, the only desire being that less than 50% of the film contain thiols. It was found using ATR-IR (section 2.7.2) that a 60% thioacetate/40% acetate (v/v) silane solution mixture, (approx. 1.5 mM total silane concentration, 0.9 mM thioacetate/0.7 mM acetate, using the density of octadecyltrichlorosilane (0.984 g/mL) for each compound), produced a 25% thioacetate film on the surface. (Figure 4.1)

Instead of repeating the orientational studies to determine order in films produced on the mixed SAM surface, a more fundamental investigation into the nature of the protein-surface interactions involved with protein film formation was conducted. To this end, a set of adsorption isotherms/removal studies was obtained for protein adsorption on three surfaces - 100% thiol, 100% hydroxyl, and the mixed SAM surface. For yeast cytochrome c adsorbed on each surface, the hypothesis was that strong, multiple binding processes would exist on the 100% thiol surface, while the hydroxyl surface would have the lowest and weakest protein binding. It was hoped that on the mixed surface a strong covalent interaction would prevail, with a reduced amount of non-specific adsorption.

In addition to studying the interaction of yeast cytochrome c on these surfaces, horse heart cytochrome c was also included. As described in section 1.4.2, horse heart cyt
Figure 4.1. Idealized Organized Yeast Cytochrome c Film on a Mixed 25% Thiol-/Hydroxyl-capped Silane Self-Assembled Monolayer

Wild type yeast cytochrome c covalently bound through only the near-surface cysteine residue

Mixed 25% thiol-/hydroxyl-capped silane self-assembled monolayer

quartz or sol-gel waveguide substrate
c is very similar to yeast except it has a higher thermodynamic stability and the two proteins vary about 40% in amino acid content, including the lack of a surface cysteine residue for horse heart cyt c. It was hypothesized that horse heart cyt c would bind less strongly to each surface, with the lowest protein-surface interactions on the hydrophilic hydroxyl surface. Two reasons for the expected reduction in protein-surface interactions were that horse heart cannot covalently bind to the SAMs and its higher thermodynamic stability would render it less likely to denature on the surface. These are also the reasons for choosing to include horse heart cyt c as a part of this investigation. Due to its structural similarity to yeast cyt c, it would provide information about how differences in thermodynamic stability affect protein interaction with surfaces. The fact that one protein can covalently bind to the thiol-reactive surfaces and one cannot, gives an indication on how the possibility of binding or not affects protein film formation. This effect can also be observed with the yeast cyt c on the hydroxyl surface.

4.2. EXPERIMENTAL
4.2.1. Protein Purification

Yeast and horse heart cytochromes c were purified according to a modified version of the procedure by Brautigan et al., [104].

A CM-52 column was prepared by dissolving 35 g of CM-52 in 0.5 M phosphate buffer (pH ~ 9). After decanting the fines, more buffer was added and the pH adjusted to 7 using 5 M HCl. The CM-52 was poured into a Pharmaco column (2.5" i.d.) with flow adapters at each end. A pump (Pharmacia LKB Pump P-1) was attached to the top flow adapter and buffer was pumped through the column at a rate of 100 mL/hr until the column material was packed. After this high ionic strength treatment (i.s. >20 mS), low ionic strength buffer was pumped through at the same flow rate until the eluent had the same low ionic strength (i.s. ~ 5 mS). The protein was dissolved (35-40 mg) in 2 mL of deionized
water (i.s. = 0 mS). A small amount of dithiothreitol (for yeast cyt c only) and sodium dithionite, (< 1 mg each), was added to the solution. The protein was loaded onto the column at a rate of 50 mL/hr. The protein was then eluted from the column using a gradient consisting of two buffers: 100 mL of low concentration, LC, 35 mM phosphate buffer (i.s. 6-7 mS) and 100 mL of high concentration, HC, 150 mM (i.s. >20 mS). The gradient was 0% HC to 100% HC. The process was monitored by an ISCO V* Absorbance in-line detector set at 410 nm and fractions were collected using a Pharmacia LKB FRAC-1 fraction collector set to collect 3 mL fractions for a total of 180 mL. The collection time took 2-3 hours (pump speed at ~ 100 mL/hr) and was either performed in a cold room (4°C) or at ambient room temperature (the short collection time not necessitating cold room use). The column packing was stored at 4°C in high ionic strength buffer and sodium azide.

A typical absorbance spectrum of the fractions is presented in Figure 4.2. The protein solution comprising fractions in the latter two-thirds of the peak were collected together for use as pure protein, the rest was discarded. The usable fractions were concentrated together under nitrogen by extrusion through a YM-3 filter (Diaflo Ultrafiltration Membranes, Amicon, Inc.) which passed species less than 3000 MW. Generally, the resulting protein concentration was 150-200 mM. Before use, the protein was re-reduced with sodium dithionite (and dithiothreitol for yeast cyt c) and dialyzed in the appropriate buffer (usually 50 mM phosphate, 100 mM NaCl, pH 6.2) for 16-20 hours, changing the 800 mL of buffer (for 5-10 mL of protein solution) at least 4 times.

4.2.2. Protein Solution Preparation and Film Formation

The concentration of purified protein solutions which had been dialyzed overnight, as described in section 4.2.1, was determined by UV-Vis using a 1 mm quartz cuvet. For the isotherm studies, the concentration was calculated using the absorbance at 520 nm, (ε520
Figure 4.2. Example Protein Purification Spectrum - Absorbance vs. Collected Fraction for Yeast Cytochrome c. Absorbance monitored at 412 nm for each fraction using a 1 cm pathlength cell then plotted versus the fraction number. Protein was in a phosphate buffer, pH 7. Due to the use of a concentration gradient for protein elution, the phosphate concentration varied from 35 μM to 150 μM over the series of fractions.
= 11,700 M\(^{-1}\cdot\text{cm}^{-1}\)), for yeast cyt c solutions, and at 410 nm \((\varepsilon_{410} = 106,100 \text{ M}^{-1}\cdot\text{cm}^{-1})\) for horse heart cyt c. For the surface coverage studies, the absorbance at 412 nm \((\varepsilon_{412} = 96,000 \text{ M}^{-1}\cdot\text{cm}^{-1})\) for yeast cyt c and again at 410 nm for horse heart cyt c were used to determine concentration.

A series of solutions was prepared for each isotherm experiment, ranging from 1 µM to 60 µM. Each solution was prepared individually from the concentrated sample. For the surface coverage experiments, a solution of 30 µM protein was prepared. All solutions were prepared using 50 mM phosphate, 100 mM NaCl, pH 6.2 buffer.

4.2.3. Adsorption Isotherms and Desorption Studies

The experimental set-up for adsorption isotherms was identical to that used for absorbance linear dichroism, section 3.2.4, with a few minor changes. The substrates used were uranyl-doped waveguides which fluoresced at about 560 nm when excited at 514.5 nm. Therefore, an emission filter which passed light at 568 nm ± 35 nm, (Omega Optics, 568DF35), was attached to the lens of the CCD camera for data collection of the fluorescence which was proportional to the absorbance. Integration times were generally 10-15 seconds with an incident radiation power of 40-50 mW on the prism face.

After obtaining four pictures of the streak for the blank in each polarization, the flowcell was flushed using 2 mL of 1 µM protein solution and allowed to sit for 20 min. Before collecting pictures of the streak with the protein film, the flow cell was flushed using 4-6 mL of buffer, (50 mM phosphate, 100 mM NaCl, pH 7.2). The next concentration of protein solution, 5 µM, was then loaded into the flow cell. This procedure was followed for 10 µM, 20 µM, 30 µM, and 60 µM solutions, with data collection after each incubation/rinse. Following the isotherm, a desorption study was performed by rinsing the cell with LSB, HSB, and DTT (defined in section 3.2.3), each having 20
minute incubation times. Before each data collection, the cell was rinsed with 4-6 mL of LSB.

Data was analyzed using IPLab Spectrum software to plot the intensity of the streak versus distance of the beam traveling down the waveguide and to measure an average background intensity. This data was transferred to a spreadsheet in Microsoft Excel ("Loss Coefficient Worksheet") which was designed to average the data and determined the loss coefficient (slope) in each polarization. The values for each concentration or rinse in both polarizations were averaged together along with data from several trials. The blank loss coefficients were subtracted from the rest and an isotherm of loss coefficient vs. concentration was plotted. After correction for surface coverage, the isotherm of surface coverage vs. concentration was plotted. Desorption data was analyzed by ratioing the loss coefficient for each rinse to that for the final concentration of the adsorption isotherm. These values were also converted to monolayer fractions and plotted versus the rinse order.

4.2.4 Protein Surface Coverage

For surface coverage studies, soda-lime glass slides were cut into pieces having dimensions of 2 cm x 2.5 cm x 0.1 cm. These glass pieces were cleaned according to the procedure in section 2.3 and silanized in the appropriate silane solution with subsequent surface reduction as described in section 2.4. For silanization, eleven pieces (one "set") were placed in a 4 oz. jar containing a small Teflon disk with slots, (separate jars for each step). After drying, the pieces were placed in a clean jar and covered with protein solution prepared as described in section 4.2.2. When incubation was complete, the protein solution was pipetted off and buffer solution was added to the jar. The pieces were rinsed several times to remove excess protein. A small Teflon cup was filled with about 9 mL of 1 M NaOH/1% Triton X-100 solution (enough to cover the substrates) and the protein-coated glass pieces were transferred into it one at a time. After sonication for 20 min, the
solution was transferred by pipette to a 10-mL volumetric flask. A small amount of base solution was used to rinse the Teflon cup and slides and this was added to the flask to make 10 mL. The absorbance of the solution at 410 nm, obtained by UV-Vis in a 1 cm pathlength cell was used to determine the concentration of protein in the solution which was used to calculate the amount of yeast cyt c on the surface. The absorbance spectrum of a known concentration of protein prepared in the base solution was used to determine a molar absorptivity of 96,000 M⁻¹·cm⁻¹.

To determine the amount of protein/SAM effectively removed from the glass by the above procedure, an epifluorescence study was performed. A set of glass pieces was prepared as described above, using a 1:8 Zn:Fe yeast cyt c protein solution. The epifluorescence set-up described in section 3.2.3 was used to collect fluorescence intensities for five of the pieces after protein incubation and again after the base soak. Before data collection, multiple buffer rinses were used to remove loosely bound protein (after incubation in the protein solution) or residual protein-base solution (after the base soak). The slides were shaken to remove excess buffer, since drying with N₂ appeared to substantially alter the fluorescence intensity. Therefore, the slides were wet during data collection, but not covered by liquid as in a fluid cell. A clean, bare glass slide was used to obtain background intensity values. The relative fluorescence decrease was used to correct the surface coverage values obtained in the absorbance experiments.

4.3. RESULTS AND DISCUSSION

4.3.1. Protein Surface Coverage

a. Steady State Determination

Before determining surface coverage or obtaining adsorption isotherms for each protein on each surface with varying solution concentration, it was necessary to determine the amount of time necessary to reach steady-state for protein film formation from a given
solution concentration. Therefore, an adsorption isotherm for a 30 μM solution of yeast cyt c on a mixed SAM surface was obtained over 90 minutes. This isotherm (Figure 4.3) shows that a steady state is reached after 5 minutes or less. To assure that sufficient time was allowed for adsorption to occur when obtaining adsorption isotherm data, an incubation time of 20 minutes was chosen. This value was well on the plateau for the solution-film steady-state, yet short enough to be practical for data collection.

b. Method Development

Since previous rinse studies, (section 3.3.3), indicated that complete removal of protein from the self-assembled monolayer was not achieved by any single rinse nor a combination of rinses, a sodium hydroxide solution was used to remove both the protein and the SAM. Under basic conditions, silanes are fairly readily cleaved from a glass surface. By removing the SAM, effectively all of the protein would be removed as well. A small amount of a non-ionic surfactant was also added to the base solution in order to inhibit desorbed protein from adsorbing to the bare glass substrates. To assess the effect of the sodium hydroxide solution on the protein’s visible spectrum, specifically at the Soret band which would be used to determine the concentration of removed protein, a protein solution was prepared directly in this matrix. The spectrum is shown in Figure 4.4. The peak maximum of the Soret band is at 412 nm, which is the same as its position when the protein is in a phosphate buffer. The concentration of the solution prepared was calculated to be 30 μM, based on the weight amount of dry protein added to the solution. The molar absorptivity was determined to be 96,000 M⁻¹cm⁻¹ at 412 nm in the base solution.

The effectiveness of the sodium hydroxide soak on removing the protein-SAM film was determined using epifluorescence of a film formed from a 1:8 Zn:Fe yeast cyt c protein solution. It was found that 72 ± 9 % of the protein-SAM film was removed by this method. The protein concentrations determined through absorbance measurements were
Figure 4.3. Adsorption Isotherm for 30 μM Yeast Cytochrome c on Mixed SAM Surface for 90 Minute Period. This adsorption isotherm was used to establish the amount of incubation time required to reach a steady-state for protein adsorption. An incubation time of 20 minutes was chosen for concentration-based adsorption isotherms and surface coverage measurements since this point lies well on the plateau of the isotherm.
Figure 4.4. Absorbance Spectrum for 30 μM Yeast Cytochrome c in 1 M NaOH/1% Triton X-100 Solution. The absorbance at the Soret band was used to determine a molar absorptivity of $\epsilon = 96,000 \text{ M}^{-1}\text{cm}^{-1}$ based on a solution concentration of 30 μM.
corrected by this factor when determining the surface coverage. The measurements should be considered approximate owing to the many assumptions necessary to make the calculations. In addition to those stated above, the surface area was estimated from the physical area of a cut glass piece, and each piece may not have been exactly the same size. Nevertheless, repeated measurements, (each surface coverage was determined twice), gave very similar results, translating into fairly low standard deviations.

c. Yeast Cyt c

The results of the surface coverage studies are presented in Table 4.1. The quantity of protein present on each surface was converted to a fraction of a monolayer using $2.2 \times 10^{-11}$ mol/cm$^2$ as a full monolayer coverage. This value was based on the protein crystal structure dimensions of 25 x 25 x 37 Å, [105] assuming a closest-packed film with a geometrically random orientation on an atomically flat surface. It was also assumed that the proteins adsorb without conformational changes which lead to "spreading."

Yeast cyt c formed about half of a monolayer on the pure thiol, (0.55 ± 0.07 ML) and hydroxyl surfaces, (0.56 ± 0.06 ML), but only about half of that amount on the mixed surface, (0.23 ± 0.03 ML). As discussed in the introduction to this chapter, section 4.1, the hydroxyl-terminated SAM was chosen to reduce the non-specific adsorption observed with the thiol-terminated SAM. To assess the effect of the hydroxyl-terminated surface on reduction of protein adsorption, characterization of protein surface coverage, film formation and protein removal was done on a 100% hydroxyl surface as well as the 100% thiol and thiol-hydroxyl mixture. The results here indicate that the hydroxyl SAM surface used in this study does not effectively reduce protein adsorption relative to the thiol SAM surface. It is of note that yeast cyt c adsorbed equally on the thiol and hydroxyl surfaces. This observation was not anticipated for a couple of reasons. First, the choice of the hydroxyl surface for reduction (ideally, elimination) of non-specific adsorption was based upon
Table 4.1. Surface Coverages (in Fraction of a Monolayer*) for Yeast and Horse Heart Cyt c on Thiol, Hydroxyl and Mixed SAM Surfaces

<table>
<thead>
<tr>
<th>Surface</th>
<th>Yeast</th>
<th>Horse Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiol</td>
<td>0.55 ± 0.07</td>
<td>0.30 ± 0.06</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>0.56 ± 0.06</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Mixed</td>
<td>0.23 ± 0.03</td>
<td>0.39 ± 0.09</td>
</tr>
</tbody>
</table>

*Based on a complete monolayer surface coverage of $2.2 \times 10^{-12}$ mol/cm$^2$. 
characterization of protein films formed on hydroxyl-terminated alkylthiol SAMs \[108\] in which a reduction of protein adsorption was observed. The lower adsorption was attributed to the hydrophilicity of the hydroxyl SAM surface. However, the hydroxyl alkylsilane SAMs used in the present study were shown in section 2.7.3 to be less hydrophilic than the hydroxyl alkylthiol SAMs, based on their water contact angles of 49°, (section 2.7.3) vs. 7-10°, \[81\], respectively. Causes for this difference are discussed in section 2.7.3. Therefore, it is possible that the surface was not sufficiently hydrophilic to produce a significant reduction in adsorbed protein.

Second, both site-specific binding and non-specific adsorption occur on the 100% thiol SAM, as discussed in section 3.3.3. On the hydroxyl surface, however, film formation is due solely to non-specific binding. (Again, non-specific refers to any non-covalent interactions between the protein and surface). Since no specific binding of the protein to the hydroxyl surface was designed, a lower surface coverage on the hydroxyl surface was expected. It is possible that the protein could be adsorbed at defect sites in the SAM film. Electrostatic interactions with the negatively charged glass substrate would be through defect sites, or possibly through the SAM, since it is only about 16 - 20 Å thick (section 2.7.4). It has been shown that silane monolayers reduce, but do not completely shield substrate charges. \[100\]. In addition, at defect sites, the hydrocarbon chain region of the SAM would be exposed, leading to hydrophobic protein-surface interactions. Also possible would be hydrogen bonding between the hydroxyl SAM and protein. This last type of interaction is not available for the protein adsorption on the thiol SAM. Protein adsorption due to hydrogen bonding interactions on the hydroxyl surface may account for the similar surface coverage to the thiol SAM surface coverage, especially since the removal results from section 3.3.3 show that the apparent amount of covalent binding of yeast cyt c on the thiol SAM is a relatively small percentage of the protein film.
Several factors may account for the observation that the amount of surface coverage on the thiol and hydroxyl SAMs is limited to about half of a monolayer. These include the incubation time, the ionic strength of the protein solution, and the extent of denaturation. The protein incubation time for these studies was only 20 minutes, much shorter than the 8 and 48 hour protein incubations used for studies reported in Chapter 3. However, a study of the adsorption over time showed a steady-state plateau was reached after about 5 minutes (section 4.3.1a). Since about the same coverage was obtained for 8 hours of incubation, if time is a factor, additional adsorption must occur at times greater than 8 hours (i.e. another rise in adsorption with an additional plateau).

As discussed in section 3.3.3, the possible difference in surface coverage between the 8 hour and 48 hour incubations was thought to be due to time or the electrolyte concentration in the incubation solution (100 mM NaCl) versus no additional salt, respectively. For the studies discussed in this chapter, the incubation solution was similar to that for the 8 hour incubations in Chapter 3. The surface coverages, (0.55 for 20 min. and 0.3 for 8 hour), are also similar, especially owing to the different procedures by which they were measured (see section 3.2.3 and section 4.2.4). Therefore, it is possible that a full monolayer was not formed on the thiol and hydroxyl SAMs for the surface coverages measured after a 20 minute protein incubation because the ionic strength of the protein solution prevented some of the electrostatic protein-surface interactions.

Protein “spreading” on the surface may lead to coverage of the surface with less protein than expected, based on the crystallographic dimensions of the protein. McGuire et al. [106] interpreted their kinetic data for bacteriophage T4 lysozyme mutants on hydrophilic and hydrophobic surfaces with a mechanism that includes two different states, in which the higher binding state represents coverage of a larger surface area by the protein. Others have also interpreted their data as indicating structural protein changes and unfolding which lead to an increase in the surface area of the protein which is in contact with the
surface [102a]. Since the calculation of a full-packed monolayer assumes the space occupied by each protein is related to its native structure, a conformational change which increases the occupied space would make it appear as though less of the surface were covered. However, the surface-bound protein conformation was not addressed in this study.

On the mixed surface, yeast cytochrome c adsorbed to only a quarter of a monolayer. This result does not follow from the surface coverages obtained on the 100% thiol and 100% hydroxyl SAM surfaces. Based on the surface coverage of 0.56 ML on the 100% hydroxyl SAM surface, this functionalized surface did not reduce protein adsorption relative to the thiol surface (0.55 ML surface coverage). Therefore, using it to "dilute" the thiol-terminated chains, forming a mixed SAM, would not necessarily reduce non-specific adsorption. There are a couple of scenarios which may explain the unique protein adsorption behavior on the mixed surface compared to the single component SAM surfaces. First, it is possible that the combination of long and short alkyl chains (C-16 thiol and C-11 hydroxyl) provides a more "fluid" surface which leads to less adsorbed protein. Second, the lower adsorption could also be due to denaturing of the protein on the surface, which would reduce the amount of protein necessary to cover the surface as described above. If this is the case, then denaturing occurs to a much greater extent on the mixed surface than for the pure surfaces. The mixed surface may create a less ordered film with more defect sites which induce stronger adsorption leading to increased denaturing. However, it is unlikely that the amount of protein represented by a quarter of a monolayer is enough to cover the surface through denaturing, especially since it would be expected that a denatured layer of protein would attract additional protein adsorption (i.e. cause multilayer coverage).
d. Horse Heart Cyt c

The surface coverages for horse heart cyt c on each surface are listed in Table 4.1. It forms approximately a third of a monolayer on the thiol, (0.30 ± 0.06 ML), and mixed, (0.39 ± 0.09 ML), surfaces and about half that amount, (0.17 ± 0.02 ML), on the hydroxyl surface. The concept of the effect of hydrophobicity/hydrophilicity on protein adsorption as described in section 4.3.1.c can be used to interpret the results obtained with horse heart cyt c. Both the thiol and mixed layers are relatively hydrophobic based on contact angle measurements, (70° and 69°, respectively; section 2.7.3), and are expected to induce higher protein adsorption than the more hydrophilic hydroxyl surface [102], (49°, section 2.7.3). Several types of protein-surface interactions are discussed above (section 4.3.1.c) to explain the formation of yeast cyt c films on each of these surfaces. The same interactions apply for horse heart cyt c, with one exception. Horse heart cyt c does not contain a surface cysteine residue (section 1.4) and therefore cannot covalently bind to the thiol or mixed SAM surfaces. This may partially account for the lower adsorption of horse heart on the thiol SAM surface relative to the yeast cyt c.

The discrepancy between the surface coverage trends for yeast versus horse heart cyt c could be related to the difference in thermodynamic stability. Since horse heart is about 15 kJ/mol more stable than yeast, it is less likely to denature as readily (section 1.4). Thus, if the process for adsorption on a surface induces denaturation of yeast cyt c, regardless of the surface type, the extent to which yeast denatures may dominate the more subtle surface characteristics. Horse heart cyt c, on the other hand, is not as prone to denaturing and, therefore, the effects of the different surface characteristics on adsorption may predominate.

The surface coverages of horse heart cyt c on all three surfaces were less than a monolayer. A couple of explanations for submonolayer film formation were discussed in section 4.3.1.c. In addition to those possibilities, for horse heart cyt c, the thermodynamic
stability may also play a role in reducing the amount of non-specific adsorption on the surfaces. However, no types of specific adsorption were designed for this system, so the variation in surface coverages between each SAM is due to variations in the degree and type of non-specific interactions possible, (electrostatic, hydrophobic, hydrogen bonding, etc.).

4.3.2. Adsorption Isotherms

a. Data Handling and General Interpretation

The adsorption isotherms for yeast cyt c on each of the SAM surfaces are shown in Figures 4.5 and 4.6. Those for horse heart cyt c on each surface are given in Figures 4.7 and 4.8. The isotherms are plotted as fractions of a monolayer versus the solution concentration. The measured loss coefficients at each solution concentration were corrected for background by subtracting a blank measurement of the loss coefficient due to the waveguide with buffer in the flowcell. Mean values of loss coefficients were calculated by averaging together loss coefficients from at least three separate trials, except for the low (0-0.5 $\mu$M) concentrations, in which only one trial of each protein on each surface was performed. A data set was not used when some or all of the values caused large standard deviations, but did not significantly affect the average value. The average corrected loss coefficients were then normalized to the value for the 30 $\mu$M protein solution, which was the solution concentration used for the surface coverage measurements, and converted to fractional surface coverage values based on the surface coverage (in monolayer units) measured for each system. (Values listed in Table 4.2.)

Qualitatively, each of the isotherms can be described as having at least two regions with differing binding processes. The rapid rise in surface coverage for the region from 0 to 1 $\mu$M solution concentration indicates high binding affinities, while the more gradual rise and leveling-off of surface coverage at the higher solution concentrations (5-60 $\mu$M) signifies a lower affinity binding process. These observations are supported by fitting each
Figure 4.5. Adsorption Isotherms for Wild Type Yeast Cytochrome c on Silane SAM Surfaces. The data points for 1 μM to 60 μM represent averages of measurements from at least 3 different samples. Error bars (not shown) are about ± 0.1 monolayer. The data points for 0 μM to 0.5 μM represent only one experiment on each SAM surface. Monolayer coverage is based on a full monolayer being $2.2 \times 10^{-11}$ mol/cm².
Figure 4.6. Adsorption Isotherms for Wild Type Yeast Cytochrome c on Silane SAM Surfaces at Low Solution Concentrations. The data points represent only one experiment on each SAM surface. Monolayer coverage is based on a full-packed monolayer being $2.2 \times 10^{-11}$ mol/cm$^2$.

a) Wild Type Yeast Cyt c on Thiol SAM Surface - Low Concentration Adsorption Isotherm

b) Wild Type Yeast Cyt c on Hydroxyl SAM Surface - Low Concentration Adsorption Isotherm

c) Wild Type Yeast Cyt c on Mixed SAM Surface - Low Concentration Adsorption Isotherm
Figure 4.7. Adsorption Isotherms for Horse Heart Cytochrome c on Silane SAM Surfaces. The data points for 1 μM to 60 μM represent averages of measurements from at least 3 different samples. Error bars (not shown) are about ± 0.1 monolayer. The data points for 0 μM to 0.5 μM represent only one experiment on each SAM surface. Monolayer coverage is based on a full monolayer being $2.2 \times 10^{-11}$ mol/cm$^2$.

a) Horse Heart Cyt c on Thiol SAM Surface - Adsorption Isotherm

b) Horse Heart Cyt c on Hydroxyl SAM Surface - Adsorption Isotherm

c) Horse Heart Cyt c on Mixed SAM Surface - Adsorption Isotherm
Figure 4.8. Adsorption Isotherms for Horse Heart Cytochrome c on Silane SAM Surfaces at Low Solution Concentrations. The data points represent only one experiment on each SAM surface. Monolayer coverage is based on a full-packed monolayer being $2.2 \times 10^{-11} \text{ mol/cm}^2$.

a) Horse Heart Cyt c on Thiol SAM Surface - Low Concentration Adsorption Isotherm

b) Horse Heart Cyt c on Hydroxyl SAM Surface - Low Concentration Adsorption Isotherm

c) Horse Heart Cyt c on Mixed SAM Surface - Low Concentration Adsorption Isotherm
Table 4.2. Adsorption Isotherm Values (in Fraction of a Monolayer*) for Yeast and Horse Heart Cyt c on Thiol, Hydroxyl and Mixed SAM Surfaces

<table>
<thead>
<tr>
<th>Protein Concentrationa</th>
<th>Ycc&lt;sup&gt;b&lt;/sup&gt; on Thiol</th>
<th>Ycc on Hydroxyl</th>
<th>Ycc on Mixed</th>
<th>HHcc&lt;sup&gt;b&lt;/sup&gt; on Thiol</th>
<th>HHcc on Hydroxyl</th>
<th>HHcc on Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.01 µM</td>
<td>0.1</td>
<td>0.02</td>
<td>0.009</td>
<td>0.05</td>
<td>0.01</td>
<td>0.0</td>
</tr>
<tr>
<td>0.05 µM</td>
<td>0.2</td>
<td>0.02</td>
<td>0.04</td>
<td>0.07</td>
<td>0.03</td>
<td>0.2</td>
</tr>
<tr>
<td>0.1 µM</td>
<td>0.3</td>
<td>0.1</td>
<td>0.07</td>
<td>0.1</td>
<td>0.04</td>
<td>0.3</td>
</tr>
<tr>
<td>0.5 µM</td>
<td>0.3</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td>0.05</td>
<td>0.4</td>
</tr>
<tr>
<td>1 µM</td>
<td>0.3 ± 0.07</td>
<td>0.3 ± 0.1</td>
<td>0.12 ± 0.03</td>
<td>0.21 ± 0.07</td>
<td>0.06 ± 0.03</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>5 µM</td>
<td>0.33 ± 0.09</td>
<td>0.3 ± 0.1</td>
<td>0.16 ± 0.04</td>
<td>0.24 ± 0.08</td>
<td>0.10 ± 0.03</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>10 µM</td>
<td>0.39 ± 0.08</td>
<td>0.4 ± 0.1</td>
<td>0.17 ± 0.04</td>
<td>0.27 ± 0.09</td>
<td>0.13 ± 0.03</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>20 µM</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.21 ± 0.05</td>
<td>0.29 ± 0.09</td>
<td>0.25 ± 0.16</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>30 µM</td>
<td>0.6 ± 0.1</td>
<td>0.56 ± 0.08</td>
<td>0.23 ± 0.04</td>
<td>0.30 ± 0.09</td>
<td>0.17 ± 0.03</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>60 µM</td>
<td>0.6 ± 0.2</td>
<td>-</td>
<td>-</td>
<td>0.3 ± 0.1</td>
<td>0.22 ± 0.11</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

a) Based on a complete monolayer surface coverage of $2.2 \times 10^{-12}$ mol/cm$^2$.
b) Only one sample was analyzed for each data point in this region (except for the 1 µM protein concentration, which was a part of both sets of experiments), therefore standard deviations cannot be reported.
c) At least three samples were analyzed to provide an average value for each data point.
d) Solution concentration values.
e) Ycc - yeast cyt c; HHcc - horse heart cyt c.
region to a Langmuir model to obtain apparent binding constants, which are listed in Table 4.3.

The Langmuir isotherm is of the form:

$$\theta = \frac{a \cdot C}{(1 + a \cdot C)}$$  \hspace{1cm} (1)

where $\theta$ is the fractional surface coverage, $a$ is the association or binding constant, and $C$ is the solution concentration [102a,107]. This model makes several assumptions, including, [102a]:

- the energy of adsorption is a constant and is not affected by the extent of surface coverage (implying that the surface is uniform).
- there are no interactions between molecules and they do not move in plane of surface (site localization).
- the adsorption is reversible (equilibrium process).
- the maximum surface coverage possible is one monolayer.

The first three assumptions do not hold well for the systems under investigation in this study. However, fitting the curves to this isotherm model does allow for some semi-quantitative comparison among the protein-surface combinations. The binding constant values obtained are not accurate, but can be considered relative values for comparison within this study. (Table 4.3)

An attempt was made to fit the entire adsorption using the Langmuir model, however unsatisfactory results were obtained. It was found that better fits of the data could be obtained by dividing the isotherms into two regions - the low concentration (0 - 1 $\mu$M) and the high concentration (5 - 60 $\mu$M) regions. For each protein on each surface, a higher binding affinity constant described the low concentration region, while the higher solution concentration region exhibits a lower binding affinity by about an order of magnitude. A possible explanation for this two-phase adsorption process is that upon protein introduction
### Table 4.3. Relative “Binding Affinities” for Yeast and Horse Heart Cyt c on Thiol, Hydroxyl and Mixed SAM Surfaces

<table>
<thead>
<tr>
<th></th>
<th>Yeast Cyt c (µM⁻¹)</th>
<th>Horse Heart Cyt c (µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-1 µM Soln. Conc.</td>
<td>5-60 µM Soln. Conc.</td>
</tr>
<tr>
<td>Thiol SAM</td>
<td>0.7 ± 0.4ᵃ</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Hydroxyl SAM</td>
<td>0.34 ± 0.04</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Mixed SAM</td>
<td>0.19 ± 0.05</td>
<td>0.013 ± 0.005</td>
</tr>
<tr>
<td>Thiol SAM</td>
<td>0.4 ± 0.1</td>
<td>0.012 ± 0.008</td>
</tr>
<tr>
<td>Hydroxyl SAM</td>
<td>0.07 ± 0.02</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>Mixed SAM</td>
<td>0.8 ± 0.5</td>
<td>0.02 ± 0.01</td>
</tr>
</tbody>
</table>

ᵃ) Standard deviation obtained from fitting program in Origin 4.1.
to the surface, it encounters a heterogeneous surface containing high affinity sites, such as defects in the SAM surface or covalent attachment. Once these defect sites are occupied, the protein then fills in the regions of lower affinity. Similar adsorption isotherms were obtained by Xie et al. [40] for horse heart cyt c adsorbed on a tentacle-type cation exchanger and their interpretation of the data is that different binding regions are observed in the isotherms due to protein binding on a heterogeneous surface.

It is possible that other models may fit the acquired data better than the Langmuir model, through consideration of factors such as irreversible binding and cooperativity. Several models have been proposed for protein adsorption on homogeneous and heterogeneous surfaces [102a,109-112]. Johnson et al. applied a Temkin isotherm model to fit adsorption isotherms for heterogeneous protein adsorption on derivatized substrates consisting of random distributions of binding functionalities [109]. While a bi-Langmuir isotherm suitably fit the data, modeling with the Temkin isotherm produced results which were more readily comparable among isotherms. However, the Temkin model assumes a uniform distribution of binding energies, which is not always accurate.

Heimburg and Marsh found that their cytochrome c binding data was best modeled by a modified Langmuir isotherm. The effects of binding to non-localized sites and the presence of non-ideal protein-protein interactions were included, [110], in addition to electrostatic binding. A series of isotherms for cyt c binding to a negatively charged lipid surface under varying ionic strengths was successfully characterized with the modified isotherm model to show an inverse relationship between binding strength and ionic strength. Initial ligand binding constants under varying ionic strengths must be determined in order to obtain the necessary parameters to fit the isotherms. This model is therefore relatively specific for adsorptions of charged species on charged surfaces. In addition, since the basis for this isotherm is the Langmuir model, assumptions such as reversibility, would still be required. As these examples and others [111-112] indicate, modeling of
adsorption isotherms is currently very specific for the type of molecular assembly under investigation. To date there is no one model available which can adequately describe protein film formation on a general level. It was not within the scope of this project to determine a model for describing the data for these specific protein-modified substrate combinations. The simple Langmuir isotherm model was chosen to semi-quantitatively compare the different systems investigated, since other models would not necessarily be significantly more appropriate. Since it was found that each data set (for a single protein-SAM combination) could be described by splitting it into two regions, each with a separate affinity constant, the sum of these two Langmuir isotherm expressions could be used to fit the complete isotherm. However, the fitting program, Origin, which was used to analyze the data did not contain an expression for a bi-Langmuir (or multi-Langmuir) isotherm. It should be noted that by separating the two regions for fitting purposes, competition between types of binding sites was not taken into account. A sum of the Langmuir isotherms describing the low and high affinity binding regions would require that the dynamics of multiple binding possibilities be considered. Therefore, since the individual constants in each binding region did not vary significantly between protein-surface sets, it was not expected that determination of an overall binding constant for each sample would provide for any addition comparisons to be made.

b. Horse Heart Cyt c

The adsorption isotherms for horse heart cyt c are shown in Figures 4.7 (whole isotherm) and 4.8 (low concentration region). The numerical data is tabulated in Table 4.2. From the complete isotherm, Figure 4.7, for each surface, it can be observed that there is an initial rapid rise in adsorption with respect to protein concentration in solution. This is followed by a slower increase in adsorption with increased concentration. A detailed look
at the low concentration region for each surface shows that the initial concentration rise does occur quickly at sub-micromolar protein concentrations.

The two-phase behavior described by the isotherm indicates a change in binding affinities over the course of the adsorption isotherm. Fits of the low concentration and high concentration regions produced the relative binding affinities listed in Table 4.3 and the fits are shown in Appendix E, Figures E.4-6. Horse heart cyt c adsorption to each of the three surfaces produces very similar binding affinities in the higher solution concentration, both to each other and to those for yeast cyt c. However, in the low concentration region, the affinity constants appear to vary between surfaces. Since the values obtained for the binding affinities are only approximate, these differences may not be significant. As discussed earlier, the Langmuir isotherm model is not accurate for these systems due to several poor assumptions that are made about the data. Thus, even though the numbers are considered relative values to each other, the model may "fit" some of these sets of data better than others. The differences do vary in a systematic way. A relatively low affinity is obtained for horse heart cyt c adsorbed to the hydroxyl surface compared to the other two modified substrates. The value of 0.07 μM⁻¹ resembles the values obtained in the low affinity region, which indicates that the protein has a very low tendency to bind to the hydroxyl surface overall. The low surface coverage obtained is supported by this interpretation.

Higher affinity constants describe adsorption of horse heart cyt c on the thiol and mixed surfaces, compared to that for the hydroxyl surface. These results are consistent with reports that proteins tend to bind less readily on hydrophilic surfaces than on hydrophobic surfaces [43, 109]. In addition, it was shown in Chapter 2 that the hydroxyl SAM surface appeared more homogenous than the thiol or mixed surfaces. The thiol surface was shown by AFM to be rougher with island-type formations compared to the smooth hydroxyl surface, (section 2.7.5). Ellipsometry measurements indicated possible
multilayer film formation for mixed layers, (section 2.7.4b). Therefore, these two surfaces may contain more heterogeneity than the hydroxyl surface, leading to more pronounced two-phase adsorption isotherms (Figures 4.6a and 4.6c), with relatively high initial binding affinities.

c. Yeast Cyt c

The adsorption isotherms for yeast cyt c are shown in Figures 4.5 (whole isotherm) and 4.6 (low concentration region). The numerical data is tabulated in Table 4.2. Again, the trend in the isotherms shows an initial quick rise in protein adsorption followed by a slower rise adsorption at higher concentrations. The binding constants for each region on each surface are listed in Table 4.3, with the fits shown in Appendix E, Figures E.1-3. In contrast to horse heart cyt c, the affinity constants in the low concentration region do not appear to significantly vary from surface to surface for yeast cyt c adsorption. (Table 4.3) This difference between the data for horse heart cyt c and yeast cyt c is consistent with the lower thermodynamic stability of yeast cyt c by 15 kJ/mol [65]. As discussed in section 4.3.1.d, yeast cyt c is more readily denatured than horse heart cyt c and this event may dominate the adsorptive process, causing the interaction on each surface to appear equivalent. However, it should be noted that the adsorption of yeast cyt c on the hydroxyl surface does follow a more gradual rise in the low concentration region, than on the other two surfaces. This may be a result of the relatively higher hydrophilicity of the hydroxyl SAM compared to the other two SAMs.

4.3.3. Desorption Studies

After the isotherm data was collected, the final protein film formed was rinsed with a series of solutions to determine the type of protein-surface interactions holding the protein on the surface. The desorption plots are given in Figures 4.9 and 4.10 for yeast and horse heart cyt c, respectively. Table 4.4 lists the numerical data used in the plots. For the
Figure 4.9. Desorption of Wild Type Yeast Cytochrome c from Silane SAM Surfaces. Measurements from at least 3 different samples were averaged together for each point. Monolayer coverage is based on a full-packed monolayer being $2.2 \times 10^{-11} \text{ mol/cm}^2$. The rinses are: LSB - low salt buffer - 50 mM phosphate, 100 mM NaCl, pH 7.2; HSB - high salt buffer - 50 mM phosphate, 100 mM NaCl, 200 mM KCl, pH 7.2; DTT - 5 mM dithiothreitol in 50 mM phosphate, 100 mM NaCl, 200 mM KCl, pH 7.2.
Figure 4.10. Desorption of Horse Heart Cytochrome c from Silane SAM Surfaces. Measurements from at least 3 different samples were averaged together for each point. Monolayer coverage is based on a full-packed monolayer being $2.2 \times 10^{-11}$ mol/cm$^2$. The rinses are: LSB - low salt buffer - 50 mM phosphate, 100 mM NaCl, pH 7.2; HSB - high salt buffer - 50 mM phosphate, 100 mM NaCl, 200 mM KCl, pH 7.2; DTT - 5 mM dithiothreitol in 50 mM phosphate, 100 mM NaCl, 200 mM KCl, pH 7.2.
Table 4.4. Removal of Yeast and Horse Heart Cyt c from Thiol, Hydroxyl and Mixed SAM Surfaces

<table>
<thead>
<tr>
<th>Rinse Type</th>
<th>Ycc$^a$ on Thiol</th>
<th>Ycc$^a$ on Hydroxyl</th>
<th>Ycc$^a$ on Mixed</th>
<th>HHcc$^a$ on Thiol</th>
<th>HHcc$^a$ on Hydroxyl</th>
<th>HHcc$^a$ on Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSB-1$^a$</td>
<td>0.6 ± 0.2</td>
<td>0.56 ± 0.08</td>
<td>0.23 ± 0.04</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>LSB-2$^a$</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.21 ± 0.04</td>
<td>0.28 ± 0.09</td>
<td>0.19 ± 0.04</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>HSB$^b$</td>
<td>0.6 ± 0.1</td>
<td>0.50 ± 0.08</td>
<td>0.17 ± 0.04</td>
<td>0.28 ± 0.09</td>
<td>0.19 ± 0.04</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>DTT$^c$</td>
<td>0.5 ± 0.1</td>
<td>0.40 ± 0.08</td>
<td>0.12 ± 0.05</td>
<td>0.25 ± 0.08</td>
<td>0.08 ± 0.03</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

a) LSB is low salt buffer, consisting of 50 mM sodium phosphate and 100 mM NaCl at pH 7.2. Two sequential rinses with this buffer were done.
b) HSB is high salt buffer, consisting of 50 mM sodium phosphate, 100 mM NaCl, and 200 mM KCl at pH 7.2.
c) DTT is a 5 mM dithiothreitol solution prepared in high salt buffer.
d) Ycc - yeast cyt c; HHcc - horse heart cyt c.
adsorption isotherms, after each incubation step, the protein solution was rinsed from the flowcell with a buffer solution. However, data was obtained right after rinsing, (a period of 2-4 minutes), directly followed by injection of the next protein solution. Therefore, the first rinse for the desorption studies was a repeat of this buffer rinse, this time including a 20 minute static incubation in the buffer to allow loosely bound protein to desorb and diffuse into the rinse solution. This buffer rinse was termed a low salt buffer (LSB) with a salt concentration of 100 mM NaCl - this was the same buffer rinse used after each protein incubation except the pH was increased from 6.2 to 7.2. This rinse removed a small portion of protein from the surface, indicating that the quick rinses after each protein incubation in the adsorption isotherm may not have allowed for complete removal of the loosely associated protein. However, within the error associated with these experiments, the amount of protein removed in this rinse is insignificant.

The second rinse consisted of a higher ionic strength solution (HSB), where KCl was added to the LSB solution to a concentration of 200 mM. The increase in ionic strength was intended to interrupt electrostatic binding and remove protein bound through charge association. Again a small portion of protein for each system was removed with this rinse, however within one standard deviation, the amount was insignificant.

The third rinse was a combination of the high salt buffer and dithiothreitol, (DTT). DTT is a disulfide reducing agent which was chosen to remove covalently bound protein. Covalent binding schemes were only possible for the yeast cyt c on the thiol and mixed surfaces, however for completeness, the final rinse was done on all systems. It was found that DTT removed some of the film, even for the protein-SAM combinations where no covalent binding was possible. The removal of the non-covalently bound protein by DTT indicates that DTT acts as a competing adsorbate to displace the protein. This hypothesis is reasonable, considering the structure of DTT, (Figure 4.11), and its high concentration (5 mM) in the rinse solution compared to the amount of protein on the surface, (about 0.1
Figure 4.11. Structure of Dithiothreitol
Therefore, the removal of protein through a DTT rinse does not necessarily indicate the protein was covalently bound.

Within one standard deviation, however, the amount of protein removed by DTT was not significant, compared to the HSB rinse, except for the yeast cyt c on the mixed SAM and for the horse heart cyt c on the hydroxyl surface. These two systems were the two with the least protein surface coverage and the lowest binding affinities, both in the low and high concentration regions of the adsorption isotherms. For the horse heart cyt c on the hydroxyl surface, the low protein surface coverage, low binding affinity constants and significant protein removal, compared to other systems, can be attributed to the hydrophilicity of the surface and thermodynamic stability of the protein. The observations made for yeast cyt c on the mixed SAM are less easily explained. The mixed surface is as hydrophobic as the thiol surface, from which the yeast cyt c was not significantly removed. Neither does increased hydrophilicity appear to affect the extent of protein-surface interactions, since yeast cyt c binds with similar affinity to the hydroxyl surface as to the thiol and mixed SAMs, with significant coverage and low removal. The yeast cyt c film appears to have a significant portion of loosely-bound protein (about half the coverage) covering the mixed SAM surface, based on the amount of protein adsorbed in the high concentration region of the adsorption isotherm, (Figure 4.5c). This is inconsistent with the concept presented earlier that the low surface coverage on this SAM results from protein denaturing and spreading, because a denatured protein would be expected to bind at multiple sites and be more difficult to remove. Therefore, there must be something unique about this protein-surface combination which leads to a relatively low coverage with a significant portion of loosely-bound protein.

It was considered that each rinse solution could be just removing loosely bound protein which would be removed as easily with a succession of the same rinse. This possibility was investigated for yeast cyt c on a mixed SAM surface and the results are
plotted in Figure 4.12. There does not appear to be significant decrease in the amount of protein over the course of 4 low salt buffer, LSB, rinses and one high salt buffer, HSB, rinse. It was therefore assumed that the amount of protein removed during a single 20 minute incubation is the amount of protein removable by the specific rinse - LSB, HSB or DTT.

4.4. CONCLUSIONS

The set of studies described above was chosen to investigate the nature of protein-surface interactions for yeast and horse heart cytochromes c on three silane self-assembled monolayer modified surfaces. It was found that each protein interacted differently with each surface, with no distinct pattern. Some generalities that may be drawn from the results are that the proteins adsorbed less on the more hydrophilic single component surface than on the hydrophobic single component surface. The combination of silanes to form a mixed surface created a surface with a very different set of properties from either of the single component SAMs, as indicated by the unpredictable and difficult to interpret results obtained on this surface with each protein. A significant degree of non-specific and irreversible binding occurred for all systems. This indicates that for the mixed surface, the goal of producing a surface which would increase the amount of site-directed covalent binding (through disulfide bond formation) and decrease other protein-surface interactions was not met.
Figure 4.12. Repeated Rinses for Removal of Yeast Cytochrome c from Mixed SAM Surface. This series of rinses was used to determine the efficiency of a single rinse to remove advantageously adsorbed protein from the surface. A mixed SAM was incubated for 20 minutes in 30 μM yeast cyt c. The loss coefficient obtained from absorbance of the initial protein film was set to represent 100% of a protein film and loss coefficients obtained from subsequent rinses were ratioed to this value. The rinses are: LSB - low salt buffer - 50 mM phosphate, 100 mM NaCl, pH 7.2; HSB - high salt buffer - 50 mM phosphate, 100 mM NaCl, 200 mM KCl, pH 7.2.
CHAPTER 5

COMPARISON OF INTERACTIONS FOR A VARIANT YEAST CYTOCHROME C ON 100% THIOL-CAPPED SAMS WITH 100% HYDROXYL-CAPPED SAMS AND MIXED SAMS

5.1. INTRODUCTION

The results presented in Chapter 4 showed that the interaction of yeast cytochrome c with a mixed SAM surface was much different than with a pure thiol surface, indicating that the change in surface modification did affect the protein film formation. However, from the desorption studies, there still appeared to be a significant fraction of non-specifically bound protein on the surface. In addition, it was questionable as to what fraction, if any, of the protein film was covalently bound (section 4.3.3). It is thought that the location of the cysteine residue on wild type yeast cytochrome c may restrict disulfide bond formation with an extrinsic thiol. The cysteine residue is in a hydrophobic pocket about 5 Å inside the surface of the protein and partial unfolding of the terminal α-helix is required to allow extrinsic binding with a thiol-reactive moiety. [55] Partial unfolding of the protein reduces its stability [55]. Lower stability would possibly make it more susceptible to denaturing on the surface, thereby increasing the amount of non-specifically bound protein.

From the studies described in Chapter 4, it was found that non-specific, irreversible binding of yeast cyt c on the mixed surface decreased. There was also a small percentage increase in the apparent amount of covalently-bound material. These results may be due to increased availability of the thiol groups on the mixed surface. With less steric hindrance due to nearby thiol groups, the protein structure could have been less perturbed when binding to the surface, thus reducing protein destabilization and denaturation. Further increase in specific binding with a decrease in non-specific binding might be achieved by
moving the single reduced cysteine to a location directly on the protein surface, such that little change in conformation would be required for bonding to occur.

Many structural variants of proteins have been produced, [9,10a,17,55,66-72]. Especially useful in many studies is the ability to alter the amino acid sequence of a protein without significantly affecting the protein stability. Several different variants of yeast cytochrome c have been prepared [55,66-72]. It has been shown that many of these variants are as stable as the native form. In particular, exchanging Cys102 for Thr102 results in a more stable protein, [57], since extrinsic disulfide bond formation through the Cys102 destabilizes the protein [55]. One variant that has been prepared is a 8Thr102/102Cys8 variant of iso-1-saccharomyces cerevisiae (yeast cyt c), where the threonine at position 8 (on the protein surface) was replaced with a cysteine, and the cysteine at 102 was replaced with a threonine. (Figure 5.1) Essentially, this moved the thiol-reactive moiety to a different position, possibly making it more available for binding at that site. In addition, by changing the location of the site at which disulfide binding to an extrinsic thiol can occur, relative to the position of the heme group, the measured molecular orientation of the protein relative to the surface would be altered, if a film were formed solely via bonding to Cys8.

This chapter will describe the investigation of variant yeast cyt c film formation on and removal from pure thiol, pure hydroxyl and mixed thiol/hydroxyl SAMs. Comparison of the results will be made to those of wild type yeast cyt c and horse heart cyt c. It is expected that the variant should behave fairly similarly to the native protein, except to show an increased stability in film formation (indicated by reduced non-specific binding) and increased site-specific binding (indicated by a greater portion of protein removal by a disulfide reducing agent).
Figure 5.1. X-Ray Crystal Structure of Wild Type Yeast Cytochrome c
(Threonine in position 8 - location of Cysteine in Variant)

Threonine in position 8 is replaced by Cysteine in the Variant.
5.2. EXPERIMENTAL

5.2.1. Protein Solution Preparation and Film Formation

The variant yeast cyt c, 8Thr102/102Cys8, was obtained from A. G. Mauk’s lab at the University of British Columbia and was provided as a 5.35 mM solution with 5 mM dithiothreitol to prevent dimer formation. The solution was stored in a liquid nitrogen dewar until use. A small aliquot (usually about 8-10 µL) was removed and diluted to about 100 µM in a 50 mM phosphate buffer, 100 mM NaCl, pH 6.2. A small amount of sodium dithionite (<1µg) was added to reduce the iron and this solution was dialyzed in the same buffer solution for 16-20 hours, changing the 800 mL of buffer (for 5-10 mL of protein solution) at least 4 times, in order to remove the dithiothreitol and dithionite. The concentration was determined by absorbance at 410 nm, using $\varepsilon_{410} = 106,100 \text{ M}^{-1}\text{cm}^{-1}$. A series of solutions was prepared for each isotherm experiment, ranging from 1 µM to 60 µM. Each solution was prepared individually from the concentrated sample. For the surface coverage experiments, a solution of 30 µM protein was prepared, except for the surface coverage experiments on the thiol surface, in which a 20 µM solution was used. All solutions were prepared using 50 mM phosphate, 100 mM NaCl, pH 6.2 buffer.

5.2.2. Adsorption Isotherms and Desorption Studies

Adsorption isotherms and removal data were obtained for the variant yeast cyt c on each surface according to the same procedures described in section 4.2.3.

5.2.3. Protein Surface Coverage

Surface coverages for variant yeast cyt c on each surface were determined in the manner described in section 4.2.4. One exception to this procedure was the use of a 20 µM protein solution for the pure thiol SAM surface. In order to compare this surface coverage with the other systems, the loss coefficient values for the adsorption isotherm were normalized to
the 20 μM data point, then converted to surface coverage values. This allowed determination of the apparent surface coverage at each concentration.

5.3. RESULTS AND DISCUSSION

5.3.1. Protein Surface Coverage

The results of the surface coverage studies are presented in Table 5.1. The quantity of protein present on each surface was converted to a fraction of a monolayer using $2.2 \times 10^{-11}$ mol/cm$^2$ as a full monolayer coverage, applying the assumptions stated in section 4.3.1c. The difference in amino acid sequence between the variant and the yeast was intended to reduce steric hindrance associated with the formation of disulfide bonds and therefore improve covalent binding. One possible result would be an increase in the amount of surface coverage on the thiol-reactive surfaces. However, coverage of the variant on the thiol surface was comparable to that of wild type yeast cyt c. Coverage on the mixed SAM surface was greater than that of yeast cyt c, yet for both proteins the lowest surface coverage was on the mixed SAMs. This moderate increase in coverage over the wild type yeast cyt c could be indicative of increased disulfide bond formation. On the hydroxyl surface, however, significantly more variant adsorbed to the surface than either the yeast or horse heart cyt c. This result on a non-bonding surface would not be expected based on the small structural difference between the variant and wild type yeast cytochromes c.

It is possible that the change in amino acids decreased the protein stability, (this has not been quantitatively investigated and was not addressed in these studies), relative to the wild type, such that the protein would be more prone to non-specifically bind. Cutler et al. used spectroelectrochemistry to show that replacing the Cys102 with threonine resulted in an increased stability over the native form [69]. However, Thr8 participates in hydrogen bonding which stabilizes a distortion that appears necessary for a the Cys14 thioether bond to the heme group. Changing Thr8 to Cys8 in the variant disrupts the hydrogen bonding
Table 5.1. Surface Coverage Values (Fraction of a Monolayer) for Variant Yeast Cyt c on Each Silane SAM Surface as Compared to Wild Type Yeast and Horse Heart Cyt c

<table>
<thead>
<tr>
<th></th>
<th>Variant</th>
<th>Horse Heart</th>
<th>Yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiol</td>
<td>0.6 ± 0.1</td>
<td>.30 ± .06</td>
<td>.55 ± .07</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>0.73 ± 0.09</td>
<td>.17 ± .02</td>
<td>.56 ± .06</td>
</tr>
<tr>
<td>Mixed</td>
<td>0.39 ± 0.05</td>
<td>.39 ± .09</td>
<td>.23 ± .03</td>
</tr>
</tbody>
</table>

* Based on a complete monolayer surface coverage of $2.2 \times 10^{-12}$ mol/cm$^2$. 
interaction (especially when extrinsic bonding occurs) and may destabilize the heme binding [55]. Thus, while the Cys102Thr mutation stabilizes the protein structure, the Thr8Cys mutation may result in destabilization. The combination of the two changes simultaneously could result in a cancelling-out of these effects, providing a protein with relatively similar stability to the native form.

5.3.2. Adsorption Isotherms

In general, the adsorption isotherms (Figures 5.2 and 5.3 and Table 5.2) for the variant follow the same trend as those obtained for wild type yeast and horse heart cyt c on each surface. There are two regions of adsorption, a high affinity and a low affinity binding region. The main difference is that the transition between the two regions is less defined. One exception is the adsorption of the variant on the mixed SAM, (Figure 5.2c), in which there is a steep rise in adsorption in the low concentration region (0 - 1 \( \mu M \)) which rolls over and levels off quickly in the high concentration region (5-60 \( \mu M \) protein solution concentration).

The binding affinities for each surface are about the same as those for wild type yeast and horse heart cyt c and show similar trends. (Refer to Table 5.3 for values and Appendix E, Figures E.7-9 for the fits.) In the low concentration regime, a high binding affinity constant is obtained, while an affinity constant about an order of magnitude lower describes the high concentration region. This indicates that the variant cyt c behavior during film formation is similar to that of the wild type yeast cyt c.

5.3.3. Desorption Studies

The plots for the removal of variant cyt c from each of the three SAMs are shown in Figure 5.4, (data in Table 5.4). As with the wild type yeast and horse heart cyt c films on each surface, there are only slight decreases in the amount of variant protein remaining on
Figure 5.2. Adsorption Isotherms for Variant Yeast Cytochrome c on Silane SAM Surfaces. The data points for 1 μM to 60 μM represent averages of measurements from at least 3 different samples. Error bars (not shown) are about ± 0.1 monolayer. The data points for 0 μM to 0.5 μM represent only one experiment on each SAM surface. Monolayer coverage is based on a full monolayer being $2.2 \times 10^{-11}$ mol/cm$^2$. 

a) Variant Yeast Cyt c on Thiol SAM Surface - Adsorption Isotherm 

Monolayer vs. Concentration (μM) 

b) Variant Yeast Cyt c on Hydroxyl SAM Surface - Adsorption Isotherm 

Monolayer vs. Concentration (μM) 

c) Variant Yeast Cyt c on Mixed SAM Surface - Adsorption Isotherm 

Monolayer vs. Concentration (μM)
Figure 5.3. Adsorption Isotherms for Variant Yeast Cytochrome c on Silane SAM Surfaces at Low Solution Concentrations. The data points represent only one experiment on each SAM surface. Monolayer coverage is based on a full-packed monolayer being $2.2 \times 10^{-11} \text{ mol/cm}^2$.

a) Variant Yeast Cyt c on Thiol SAM Surface - Low Concentration Adsorption Isotherm

b) Variant Yeast Cyt c on Hydroxyl SAM Surface - Low Concentration Adsorption Isotherm

c) Variant Yeast Cyt c on Mixed SAM Surface - Low Concentration Adsorption Isotherm
Table 5.2. Adsorption Isotherm Values in Fraction of a Monolayer for Variant Yeast Cytochrome c on Each SAM Surface

a) High Concentration Region

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>Variant on Thiol</th>
<th>Variant on Hydroxyl</th>
<th>Variant on Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 µM</td>
<td>0.3 ± 0.1</td>
<td>0.20 ± 0.05</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td>5 µM</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>10 µM</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>20 µM</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>30 µM</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>60 µM</td>
<td>0.7 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.39 ± 0.06</td>
</tr>
</tbody>
</table>

b) Low Concentration Region

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>Variant on Thiol*</th>
<th>Variant on Hydroxyl*</th>
<th>Variant on Mixed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.01 µM</td>
<td>0.0</td>
<td>0.0</td>
<td>0.06</td>
</tr>
<tr>
<td>0.05 µM</td>
<td>0.0</td>
<td>0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>0.1 µM</td>
<td>0.08</td>
<td>0.09</td>
<td>0.2</td>
</tr>
<tr>
<td>0.5 µM</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>1 µM</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Only one sample was analyzed for each data point in this region, therefore standard deviations cannot be reported.
### Table 5.3. Relative "Binding Affinities" for Variant Yeast Cyt c on Each SAM Surface

<table>
<thead>
<tr>
<th>Variant Yeast Cyt c ($\mu$M$^{-1}$)</th>
<th>0-1 $\mu$M Soln. Conc.</th>
<th>5-60 $\mu$M Soln. Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiol SAM</td>
<td>0.45 ± 0.06*</td>
<td>0.06 ± 0.02*</td>
</tr>
<tr>
<td>Hydroxyl SAM</td>
<td>0.29 ± 0.05</td>
<td>0.105 ± 0.005</td>
</tr>
<tr>
<td>Mixed SAM</td>
<td>0.4 ± 0.1</td>
<td>0.022 ± 0.01</td>
</tr>
</tbody>
</table>

*Standard deviation obtained from fitting program, Origin 4.1.
Figure 5.4. Desorption of Variant Yeast Cytochrome c from Silane SAM Surfaces. Measurements from at least 3 different samples were averaged together for each point. Monolayer coverage is based on a full-packed monolayer being $2.2 \times 10^{-11}$ mol/cm$^2$. The rinses are: LSB - low salt buffer - 50 mM phosphate, 100 mM NaCl, pH 7.2; HSB - high salt buffer - 50 mM phosphate, 100 mM NaCl, 200 mM KCl, pH 7.2; DTT - 5 mM dithiothreitol in 50 mM phosphate, 100 mM NaCl, 200 mM KCl, pH 7.2.

a) Desorption of Horse Heart Cyt c from Thiol SAM Surface

b) Desorption of Horse Heart Cyt c from Hydroxyl SAM Surface

c) Desorption of Horse Heart Cyt c from Mixed SAM Surface
Table 5.4. Removal of Variant Yeast Cyt c from Each SAM Surface

<table>
<thead>
<tr>
<th>Rinse Type</th>
<th>Variant on Thiol</th>
<th>Variant on Hydroxyl</th>
<th>Variant on Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSB-1*</td>
<td>0.6 ± 0.2</td>
<td>0.86 ± 0.02</td>
<td>0.39 ± 0.07</td>
</tr>
<tr>
<td>LSB-2*</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>HSB&quot;</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>DTT*</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>0.34 ± 0.07</td>
</tr>
</tbody>
</table>

a) LSB is low salt buffer, consisting of 50 mM sodium phosphate and 100 mM NaCl at pH 7.2. Two sequential rinses with this buffer were done.
b) HSB is high salt buffer, consisting of 50 mM sodium phosphate, 100 mM NaCl, and 200 mM KCl at pH 7.2.
c) DTT is a 5 mM dithiothreitol solution prepared in high salt buffer.
the surface after each of the following rinses: low salt buffer, (LSB), high salt buffer, (HSB) and dithiothreitol, (DTT). There is a more pronounced removal on the thiol and hydroxyl surfaces than on the mixed SAM for the variant, most likely a result of the low amount of protein adsorbed on the mixed SAM surface in the low affinity region. In contrast to the removal results for wild type yeast cyt c from the mixed SAM, in which half of the protein coverage was removed by the rinses, removal of the variant from the mixed SAM showed the smallest relative reduction in coverage compared to its removal from the other two surfaces. The same trend is seen with the variant cyt c as with the other two proteins, in that the amount of protein removed by the rinse series is approximately equivalent to the amount that is adsorbed at high solution concentration (low affinity binding).

5.4. CONCLUSIONS

Similar general adsorption and removal trends are observed for the variant cyt c on each SAM surface compared with the yeast and horse heart cyt c. Therefore, while there are some differences between the behavior of variant and wild type yeast cyt c adsorbing on each of the SAMs, each protein formed films consisting of non-specifically (and possibly some specifically) adsorbed proteins. The types of protein-surface interactions leading to film formation on each surface and reasons for submonolayer protein coverages are described in Chapter 4 (section 4.3.1). Thus, the repositioning of the unique cysteine residue to a position more available for covalent binding did not appear to increase site-directed immobilization over non-specific adsorption. This implies that the substrate modification should be altered in order to reduce the number of different affinity binding sites to one, (that for site-directed binding).
CHAPTER 6

SUMMARY AND FUTURE WORK

6.1. RESEARCH OBJECTIVE

The ability to control and characterize protein films which are designed to have a
specific orientation and functionality is of increasing concern for widespread application in
biotechnological fields such as biological sensor development, biocompatibility issues,
chromatography and nanoscale electronic devices. Since the structuring of a protein film is
expected to influence its functionality, an improved control over the type, extent and
uniformity of protein functionality in a well-ordered film is anticipated. The work
presented in this document addressed the issues of protein-surface interactions as they
related to the formation of a site-directed, immobilized protein film.

6.2. SUMMARY

6.2.1. Silane Self-Assembled Monolayers

Single component and mixed self-assembled monolayers of 1-(thioacetato)-16-(trichlorosilyl)hexadecane and 1-acetato-11-(trichlorosilane)undecane were prepared and
characterized using ATR-FTIR, ellipsometry, contact angle measurements, AFM and XPS.
ATR-FTIR and XPS results indicated the presence of thioacetate and acetate terminal
functional groups, as well as their reduction to thiol and hydroxyl groups, respectively.
Contact angle measurements indicated moderately hydrophobic SAM surfaces, except for
the moderately hydrophilic hydroxyl-terminated SAM. Deposition conditions for
monolayer coverage of single component SAMs were determined by AFM and supported
by ellipsometry measurements. Deposition conditions for the mixed SAMs were not
optimized, resulting in thickness measurements which were inconsistent with the expected trend (increasing thickness with increasing percentage of long-chained component).

6.2.2. Orientation Studies

The orientation distribution of yeast cyt c adsorbed on a pure thiol SAM was determined using a combination of IOW-ATR absorbance linear dichroism and TIRF anisotropy techniques. The unique, thiol-reactive Cys102 residue was expected to form a disulfide bond with the thiol SAM producing a site-specifically immobilized protein film with preferential orientation.

An orientation distribution of $67^\circ \pm 39^\circ$ was obtained for substrates incubated for 8 hours. A set of values could not be determined for the 48 hour incubation. However, from the data it was apparent that the protein film formed with the 48 hour incubation was distinct from the film formed with an 8 hour incubation. The broad distribution obtained for the protein film formed with an 8 hour incubation indicates a disordered film is formed under the conditions employed for film formation. Desorption studies for both the 8 hour and 48 hour samples revealed multiple types of protein-surface interactions, including electrostatic, covalent, and hydrophobic.

6.2.3. Characterization of Protein Film Formation

Yeast cyt c film formation on three SAM surfaces: 100% C-16 thiol, 100% C-11 hydroxyl, and a mixture of the two (25% thiol on the surface), was characterized by determining surface coverages, obtaining adsorption isotherms, and investigating the affect of selected rinses on protein removal. In addition, protein film formation on each SAM surface with horse heart cyt c, (which is more thermodynamically stable, but cannot form extrinsic disulfide bonds), and with a yeast cyt c variant, 8Thr102/102Cys8, was characterized and compared to native yeast cyt c. It was found that, in general, each of the
proteins behaved similarly, with minor differences attributed mainly to the larger thermodynamic stability of horse heart cyt c over the other two proteins. Surface coverages ranged from 0.25 - 0.75 monolayer formation. The adsorption isotherms for all three proteins indicated at least two binding processes characterized by a high affinity binding constant and a low affinity constant. It was proposed that the high affinity binding, which occurred at low protein solution concentration, resulted from adsorption to defect sites on the SAM surface. When these sites were filled, lower affinity binding occurred. Protein removal studies indicated that the lower affinity binding region of the adsorption isotherms was reversible, but that a significant portion of each film appeared to be irreversibly bound.

6.2.4. Final Comments

Neither the change in SAM substrate modification from pure thiol to a mixed SAM nor the “repositioning” of the thiol reactive moiety in yeast cyt c was effective in reducing non-specific binding and/or promoting covalent attachment. Therefore, the objective to form an ordered protein film by site-directed immobilization using the chosen molecular assemblies was not met. However, it was shown that employment of a site-directed immobilization strategy does not imply that an ordered film is formed, which was previously a common assumption.

6.3. FUTURE WORK

6.3.1. Silane Self-Assembled Monolayers

Upon analysis of the characterization data obtained for the mixed C-11 acetate/C-16 thioacetate SAMs, it was found that they appeared to form multilayer structures (ellipsometry) and contained defect sites (protein adsorption). Therefore, a more systematic investigation into the deposition time required to produce quality monolayer mixed SAMs is proposed in order to reduce defect sites and eliminate multilayer formation.
The integrity of the monolayer can be assessed by ellipsometry and contact angle measurements, as well as AFM. High resolution XPS may also be applied to determine relative sulfur amounts on each surface. Improvements in SAM formation are expected to affect protein adsorption on the SAM, by reducing non-specific adsorption and promoting a single, covalent interaction.

6.3.2. Site-Directed Immobilization of Proteins on SAM Modified Substrates

a. Film Formation

The mixed SAM surface was chosen to be a counterpart to LB films of DOPC doped with pyridyl disulfide-capped DOPE [20c]. Edmiston et al. characterized the orientation of wild type yeast cyt c adsorption on that surface and found that an ordered film was formed (40° ± 11°), which appeared to be primarily covalently bound (70%) [20c]. LB film formation is more controllable than silane deposition by self-assembly because an LB film is formed by pressing the molecules into a film, then transferring them to a substrate, while SAM formation relies on mainly diffusion of the molecules to the substrate where they bind to the surface in a random manner. The non-optimized conditions under which the mixed SAMs were formed may have resulted in an altered functionality due to quality of the mixed SAM. Multilayer structures with defects were formed under the conditions applied in the studies presented in Chapters 2, 4 and 5. When the conditions are optimized to produce a mixed monolayer with no multilayer formation and reduced defect sites, (as discussed in section 6.3.1), then the functionality imparted to the substrate may approach that of the LB film mixture.

Two further studies are therefore proposed. One, to investigate protein adsorption on the optimized mixed SAM. And secondly, to observe the adsorption isotherm and removal on the mixed LB film. (DOPC doped with pyridyl disulfide-capped DOPE. [20c]),
for a comparison, since only orientation and removal studies were used to investigate yeast cyt c adsorbed on the mixed LB film previously [20c].

b. Orientation Studies

The variant yeast cyt c was originally obtained for comparison with the native yeast cyt c with respect to heme orientation of the bound species. Relocation of the cysteine to position 8 was expected to change the observed heme orientation with respect to the substrate surface for a covalently-bound protein film. Since a narrow orientation distribution was obtained for the native yeast cyt c on the pyridyl disulfide-capped LB-modified substrate, it would be expected that the variant yeast cyt c would produce similar results (based on comparison of the native and variant behavior in the studies presented in this document). The altered binding site would be expected to orient the protein such that the heme tilt angle from the surface normal would be only about 10°, (estimated based on the wild type yeast cyt c crystal structure, Figure 5.1), as compared to an angle of around 40° for the native yeast cyt c.

Another orientation experiment would be to determine the orientation distribution for the variant on the thiol surface. It was assumed from the removal studies that since multiple rinses were needed to remove the protein and only about half of the protein was removable by rinses, that an ordered protein film was not formed. However, recent work by Gabbard [115] indicates that a partially ordered film may still be obtained, even when a significant fraction (50%) is not specifically bound. In addition, studies by Edmiston et al. showed that for the wild type yeast cyt c adsorbed on the pyridyl disulfide-capped LB film, about 70% of the film had a narrow distribution orientation, while 30%, which was not removed by rinsing, had a random orientation [20c]. It is proposed to measure the orientation of the variant yeast cyt c on the thiol surface before and after rinsing with
dithiothreitol, (or some disulfide reducing agent), to determine whether a combination of oriented and random subpopulations exist.

c. Conformational Studies

In order to increase understanding of what happens to a protein when forming a film at a solid-liquid interface, it would be of interest to be able to monitor the conformational state of the protein during film formation and removal. The current optical system used for the studies described in Chapters 3-5 uses a waveguide into which only one wavelength of light can be coupled at a given coupling angle. Therefore, an absorbance spectrum cannot be measured. Recently, a broadband waveguide was developed by Mendes et al. [114] which allows a fairly large range (>100 nm) of wavelengths to be simultaneously incoupled at one coupling angle. It was shown that an absorbance spectrum of a submonolayer protein film can be obtained with this waveguide [115]. Use of this optical system may aid interpretation of isotherm and removal data through information on how adsorption affects protein conformation.

d. Film Structure Versus Functionality

It has been hypothesized that the orientation of a protein electrostatically adsorbed onto an electrode affects electron transfer between the protein and electrode [116]. Spectroelectrochemical analysis can be used to investigate electron transfer of proteins in films oriented by site-directed immobilization. The use of mutants, such as the variant yeast cyt c used in the studies presented here, to alter the binding site and thereby alter protein orientation will allow the effect of orientation on protein functionality to be observed.

e. Other Studies

It was indicated through the rinse studies, that dithiothreitol may act not only to reduce disulfide bonds for removal of covalently bound protein, but it may also cause protein desorption by acting as a competing adsorbate. The implications of this observation are that removal of protein by rinsing with DTT may not indicate covalent bond formation, as many have assumed. An investigation into the use of DTT for protein removal for covalent systems versus non-covalent systems is necessary to better establish the functional properties of this molecule.

One experiment which could help to assess the ability of DTT to act as a competing adsorbate would be to prepare a protein film which is not covalently bound to the surface (such as the horse heart cyt c on the hydroxyl SAM), and rinse the surface with increasing concentrations of DTT, following the procedures described in section 4.2.3 for the desorption studies. A partner experiment would be to repeat the above desorption series on a disulfide-bound protein film. An alternate approach would be to perform a set of epifluorescence experiments, as described in 3.2.3, using fluorescein (non-binding) and SAMSA fluorescein (thiol reactive) on a thiol-capped monolayer surface.

In addition, other disulfide reducing agents are available which may be compared to dithiothreitol. Singh et al., [117], have reported a compound, N,N'-dimethyl-N,N'-bis(mercaptoacetyl)hydrazine, (DMH), which cleaves disulfides at a greater rate than DTT and may be more efficient at removal of protein covalently bound via disulfide bonds.
APPENDIX A

SYNTHETIC ROUTE FOR THIOACETATE TRICHLOROSILANES

Based on procedure described in Balachander, N. and Sukenik, C.N. Langmuir 1990, 6(11), 1621-1627.

A list of all required materials, their source and their location in the Saavedra laboratory is provided in Table A.1.

A schematic for the synthetic route is presented in Figure A.1.

A.1. Synthesis of ω-Undecenyl Mesylate

A.1.1. Preparation

Distill triethylamine - Add a small amount of CaH₂ to about 80-100 mL triethylamine in a round-bottomed flask to remove H₂O, then heat to distill. When complete, to clean up, pour the remnant with the CaH₂ slowly onto ice to slow the exothermic reaction of CaH₂ with H₂O. Store distilled triethylamine in a clean bottle at room temperature until use.

Dry a two-neck (or 3-neck), 1-L flask, a N₂ inlet, and a pressure-equalizing addition funnel in the oven. Set up as shown in Figure A.2.

A.1.2. Reaction

Combine ω-undecenyl alcohol (0.38 mol, 71.6 mL) and triethylamine (0.57 mol, 79.5 mL) in the 1-L flask. Start N₂ flow. Cool mixture in an ice bath to 0 °C. Measure out methanesulfonyl chloride (0.57 mol, 32.4 mL) in a graduated cylinder, add CH₂Cl₂ to it, and pour the mixture into the addition funnel. Add more CH₂Cl₂ until about 100 mL of CH₂Cl₂ total has been added. Allow the methanesulfonyl chloride solution to drip slowly into the flask over a period of 30 minutes, while stirring. Stir the solution for another 1.5 to 2 hours once the addition is complete. Mixture will be orange-brown in color.
Table A.1. Compound List for Silane Synthesis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Storage Location in Lab</th>
<th>Company and Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetonitrile</td>
<td>flammable cabinet</td>
<td>Mallinckrodt 0043-500 (storeroom)</td>
</tr>
<tr>
<td>ammonium chloride</td>
<td>shelf</td>
<td>Mallinckrodt 3384 (storeroom)</td>
</tr>
<tr>
<td>calcium hydride, 94%</td>
<td>shelf</td>
<td>Baker E285-05 (storeroom)</td>
</tr>
<tr>
<td>copper (II) chloride, 97%</td>
<td>desiccator</td>
<td>Aldrich 22,201-1</td>
</tr>
<tr>
<td>dibromopentane, 1,5-</td>
<td>flammable cabinet</td>
<td>Aldrich 12,800-7</td>
</tr>
<tr>
<td>dichloromethane</td>
<td>shelf</td>
<td>Mallinckrodt 3023 (storeroom)</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>flammable cabinet</td>
<td>Mallinckrodt 4992 (storeroom)</td>
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<tr>
<td>ethyl ether anhydrous</td>
<td>hood</td>
<td>Mallinckrodt 08-48 (storeroom)</td>
</tr>
<tr>
<td>hexanes</td>
<td>flammable cabinet</td>
<td>E:M HX0299-5 (storeroom)</td>
</tr>
<tr>
<td>hydrochloric acid</td>
<td>desiccator</td>
<td>Mallinckrodt H613 (storeroom)</td>
</tr>
<tr>
<td>hydrogen hexachloroplatinate (IV) hydrate, 99.9 %</td>
<td>flammable cabinet</td>
<td>Aldrich 39,832-2</td>
</tr>
<tr>
<td>isopropanol</td>
<td>shelf</td>
<td>Mallinckrodt 3032 (storeroom)</td>
</tr>
<tr>
<td>lithium aluminum hydride</td>
<td>shelf</td>
<td>Aldrich 19987-7</td>
</tr>
<tr>
<td>lithium bromide</td>
<td>shelf</td>
<td>Aldrich 21,322-5 (storeroom)</td>
</tr>
<tr>
<td>lithium chloride</td>
<td>shelf</td>
<td>Chempure 831-197 (storeroom)</td>
</tr>
<tr>
<td>magnesium sulfate anhydrous</td>
<td>shelf</td>
<td>Mallinckrodt 6070 (storeroom)</td>
</tr>
<tr>
<td>magnesium turnings</td>
<td>shelf</td>
<td>Aldrich 20,090-5</td>
</tr>
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<td>methanesulfonyl chloride</td>
<td>shelf</td>
<td>Aldrich M880-0</td>
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<tr>
<td>molecular sieve 4A</td>
<td>desiccator</td>
<td>Storeroom</td>
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<tr>
<td>potassium thioacetate, 98 %</td>
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<td>Aldrich 24,177-6</td>
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<tr>
<td>quinoline</td>
<td>shelf</td>
<td>Aldrich 24,157-1</td>
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<tr>
<td>sand</td>
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<td>Storeroom</td>
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<tr>
<td>silica gel (230-400 mesh)</td>
<td></td>
<td>Lagand Chemical Co, Inc.</td>
</tr>
<tr>
<td>sodium bicarbonate</td>
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<td>Mallinckrodt 7412 (storeroom)</td>
</tr>
<tr>
<td>sodium chloride</td>
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<td>Mallinckrodt 7581 (storeroom)</td>
</tr>
<tr>
<td>tetrahydrofuran</td>
<td></td>
<td>Aldrich 12,800-7 (Gevay's still)</td>
</tr>
<tr>
<td>TLC plates (scored 10x20 cm, 250 microns)</td>
<td></td>
<td>Analtech 02521 (storeroom)</td>
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<tr>
<td>trichlorosilane</td>
<td></td>
<td>Aldrich 17,555-2</td>
</tr>
<tr>
<td>triethyamine, 97%</td>
<td></td>
<td>Mallinckrodt 8597</td>
</tr>
<tr>
<td>α-undecenyl alcohol, 98 %</td>
<td></td>
<td>Aldrich U200-8</td>
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<tr>
<td>acetyl chloride</td>
<td></td>
<td>Aldrich 11,418-9</td>
</tr>
</tbody>
</table>
Figure A.1. Schematic of Synthetic Routes for Thioacetate and Acetate Silanes

a) Synthetic Route for C-11 and C-16 Thioacetate Silanes

Step 1

\[ \text{\(\omega\)-undecenyl alcohol} \rightarrow \text{\(\omega\)-undecenyl mesylate} \]

Step 2a

\[ \text{\(\omega\)-undecenyl mesylate} \rightarrow \text{\(\omega\)-undecenyl bromide} \]

Step 2b

\[ \text{\(\omega\)-undecenyl bromide} \rightarrow \text{\(\omega\)-hexadecenyl bromide} \]

Step 3

\[ \text{\(\omega\)-undecenyl or \(\omega\)-hexadecenyl bromide} \rightarrow \text{\(\omega\)-undecenyl or \(\omega\)-hexadecenyl thioacetate} \]

Step 4

\[ \text{\(\omega\)-undecenyl or \(\omega\)-hexadecenyl thioacetate} \rightarrow \text{1-thioacetato-11-(trichlorosilyl)undecane} \]

or

\[ \text{1-thioacetato-16-(trichlorosilyl)hexadecane} \]

b) Synthetic Route for C-11 Acetate Silanes

\[ \text{\(\omega\)-undecenyl alcohol} \rightarrow \text{\(\omega\)-undecenyl ester} \rightarrow \text{1-acetato-11-(trichlorosilyl)undecane} \]
Figure A.2. Set-up for ω-Undecenyl Mesylate Synthesis

Figure A.3. Set-up for ω-Undecenyl Bromide Synthesis

Figure A.4. TLC Results for ω-Undecenyl Bromide Synthesis
A.1.3. Clean-up

Transfer mixture to a separatory funnel and wash with cold tap water (2-3 times, using about equal H₂O:mixture), then with 5% HCl (5 mL conc. HCl added to 95 mL H₂O) - to remove excess NEt₃, then 20% NaHCO₃ (make saturated solution and dilute) - to remove excess HCl, followed by saturated aqueous NaCl. The product will be in the organic layer. Dry the solution with MgSO₄, (add until it quits clumping, but avoid adding excess, since MgSO₄ may absorb product). Gravity filter solution to remove MgSO₄ and rotovap solution to remove CH₂Cl₂. Product is a red-brown oil. Yield is usually around 75%, (actual 50 g versus theoretical 66 g, based on given quantities).

A.1.4. Purification

None at this point.
A.2. Synthesis of ω-Undecenyl Bromide

A.2.1. Preparation

Dry acetonitrile over molecular sieve for a couple of days before using.

Dry two-neck (or 3-neck), 1-L flask, pressure-equalizing addition funnel, and reflux condenser in oven. Set up as shown in Figure A.3.

A.2.2. Reaction

Combine LiBr (0.28 mol, 24.3 g) and 300 mL of acetonitrile in the flask and stir until all of the LiBr is dissolved (about 10 minutes). Charge the addition funnel with ω-undecenyl mesylate (0.14 mol, 34.7 g) mixed with 100 mL of acetonitrile. Add to the reaction over a 15 minute period. Heat the reaction at reflux (using an oil bath) for 1 hour.

Monitor the product formation by TLC. (Figure A.4) Using hexane, the bromide has an Rf of about 0.7, while the alcohol does not move, (Rf of 0). In a 1:10 ethyl acetate:hexane solution the bromide has an Rf of about 0.7 while the alcohol Rf value is near 0.5. (Do both for TLC to insure that the bromide is present, but use hexane for the column purification.) Cool to room temperature when reaction is complete.

A.2.3. Clean-up

Transfer to a separatory funnel and add about 200 mL of ether. Wash with water, 20% NaHCO₃, and saturated aqueous NaCl (about 100 mL each). Product will be in the ether phase. Dry with MgSO₄, as described in section A.1.3 and gravity filter solution. Remove ether using rotovap. Yield is usually around 50%. (Theoretical yield with given quantities is about 73 g.)
A.2.4. Purification

Pack a column with silica gel, (SiO₂, 230 - 400 mesh) by first putting a piece of cotton, then a thin layer of sand in the bottom of the column, (Figure A.5). Combine SiO₂ with hexanes for smoother packing. Make SiO₂ layer about 4 inches thick (depending on how much product is present). Add another thin layer of sand at the top (to lessen disturbance of the silica when adding the product and solvent). Flush with hexanes, then add product and allow it to go into the top part of the silica. Slowly and carefully add solvent (about twice the height of the silica packing) and run column. When complete, flush a little more hexanes through, collect this portion in a separate container and check it by TLC to assure that no product remains on the column. Rotovap to remove hexanes.

Obtain NMR of product. Compare to Figure A.6.

Note: Cleaning column - push hexane out of silica using a light flow of N₂. Dispose of dry silica in solid waste.

When preparing C-11 silanes, skip section A.3.
**Figure A.5.** Flash Chromatography Set-up

![Flash Chromatography Set-up Diagram](image)

**Figure A.7.** Set-up for w-Hexadecenyl Bromide Synthesis

*Reaction 1: Grignard Formation*

![Set-up for w-Hexadecenyl Bromide Synthesis Diagram](image)

**Figure A.8.** TLC Results for Grignard Reaction

![TLC Results for Grignard Reaction](image)
Figure A.6. $^1$H-NMR of $\omega$-Undecenyl Bromide
A.3. Synthesis of \(\omega\)-Hexadecenyl Bromide

A.3.1. Preparation

Dry a 3-neck, 100-mL flask, a pressure-equalizing addition funnel, a reflux condenser, and \(N_2\) inlet in oven overnight. Also dry a few grams of \(LiCl\) and \(CuCl_2\) in oven overnight.

a. Reaction 1: Grignard Formation

Assemble glassware as shown in Figure A.7, and add Mg turnings (125 mmol, 3 g), then dry under vacuum with a heat gun. Transfer dry THF from still to addition funnel using a syringe. Allow about 15-20 mL into flask, while stirring. Add \(\omega\)-undecenyl bromide (50 mmol, 11.6 g) to addition funnel along with 25-30 mL of THF, (50 mL of THF total). Add about 10 mL of the mixture to the flask and begin warming in an oil bath. After reaction begins (black solution), add the rest of the mixture from the addition funnel to the flask over a period of 30 minutes. Continue refluxing for 1 hour, then cool to room temperature. (If the reaction is green when it begins, water contamination is present and Grignard compound will not form.)

Check reaction by TLC to ensure Grignard formation. In hexane, the Grignard compound has an \(R_t\) of 0.68 and the bromide has an \(R_t\) of 0.5. (Figure A.8)

b. Reaction 2: Bromide Formation

Set up (Figure A.9) and flame dry a 2-neck, 500 mL flask, a pressure-equalizing addition funnel, \(N_2\) inlet and stirbar. Purge system with \(N_2\). Add 200 mL of THF and 1,5-dibromopentane (150 mmol, 25.3 g) to flask, and cool in an ice/salt bath to -10 °C. Transfer the Grignard reagent from the 3-neck flask to the addition funnel using a long-
Figure A.9. Set-up for $\omega$-Hexadecenyl Bromide Synthesis. Reaction 2: Bromide Formation

Figure A.10. Set-up for Kugelrohr Distillation

Figure A.11. TLC Results for $\omega$-Hexadecenyl Bromide Synthesis
needled syringe (20 mL disposable syringes fitted with 4-inch metal needle. may need to use multiple syringes). Add to the flask. Prepare a 0.2 M solution of LiCl (0.0152 g) and CuCl₂ (0.0484 g) in THF (1.8 mL) and add to the flask. Stir solution at -10 °C for about 12 hours.

**A.3.3. Clean-up**

Add 150 mL of ether to the flask and transfer to a separatory funnel. Wash 2 times with saturated aqueous NH₄Cl and once with saturated aqueous NaCl, (about 100 mL each). Product will be in the ether. Dry with MgSO₄, gravity filter and concentrate on a rotovap. Remove excess 1,5-dibromopentane by Kugelrohr distillation at 60 °C, use a dry ice/acetone bath beneath the collection flask to ensure collection of 1,5-dibromopentane. (Set-up, Figure A.10)

**A.3.4. Purification**

Purify by flash chromatography using hexane, (Figure A.5). Check pure product by TLC. The C₁₆Br will have an Rₜ of 0.73 and a hydrocarbon residue will have an Rₜ of 0.60 using hexane. (Figure A.11)
A.4. Synthesis of \(\omega\)-Undecenyl Thioacetate or \(\omega\)-Hexadecenyl Thioacetate

A.4.1. Preparation

Dry a 50-mL, two-neck round-bottomed flask and a reflux condenser in the oven. Set up for reflux as shown in Figure A.12.

A.4.2. Reaction

Combine 25 mL ethanol and potassium thioacetate (15 mmol, 1.71 g) in flask. Add \(\omega\)-undecenyl bromide (15 mmol, 3 g) and heat at reflux for about 2-4 hours. Monitor reaction carefully by TLC. Using hexane, the bromide has an \(R_f\) of about 0.7, while the thioacetate does not move. Using 10% ethyl acetate, the two move about the same. A solution of 5% ethyl acetate provides an \(R_f\) for the thioacetate of about 0.5. (Figure A.13)

When the reaction is complete, allow to cool to room temperature, then add 100 mL of water. Extraction of the product from the water/ethanol solution is performed using three 50-mL portions of ether. Dry the extract (combined ether portions) with MgSO_4, gravity filter and concentrate on a rotovap.

A.4.3. Purification

Use a large flash chromatography column, (to load 2-3 g), packed as described in Step 2a, for purification of compound. (Figure A.5) Use hexanes to separate reactant from product (reactant comes off column, while product remains near top of column). Change to a 10% ethyl acetate:hexanes mixture to obtain thioacetate. Collect fractions in test tubes and check by TLC. Combine pure fractions and rotovap off solvent. Check purity by TLC. (Figure A.14) If necessary, load product on a smaller flash column to further purify thioacetate from hydrocarbon impurities using 10% ethyl acetate:hexanes as the eluent.

Obtain NMR spectrum of purified product and compare to Figure A.15.
Figure A. 12. Set-up for ω-Undecenyl/ω-Hexadecenyl Thioacetate Reaction

Figure A. 13. TLC Results for Thioacetate Synthesis

Figure A. 14. TLC for Separation of Thioacetate from Hydrocarbon Residue (after separation from the bromide)
Figure A.15. $^1$H-NMR of $\omega$-Undecenyl Thioacetate
A.5. Synthesis of 1-thioacetato-11-(trichlorosilyl)undecane
(or 1-thioacetato-16-(trichlorosilyl)hexadecane)

A.5.1. Preparation

Obtain a dry 20-mL pressure tube and a magnetic stir bar.

Distill quinoline (if yellowed) over CaH₂ under vacuum at 99-100 °C. Follow the
procedure described for triethylamine distillation in section A.1.1 for clean up of excess
CaH₂. Store distilled quinoline in a foil-covered or dark bottle in the refrigerator before
use.

Distill HSiCl₃ from quinoline under N₂ at 35 °C. For 50-60 mL HSiCl₃, use 1-2
mL quinoline. **Grease all joints very well.**

Prepare a 4% solution of H₂PtCl₆·6 H₂O in isopropanol by putting 4 to 5 particles
of catalyst into about 10 mL of isopropanol. Store solution in the refrigerator, sealed and
covered in foil. Solution is good for about a year if kept from air and light. H₂PtCl₆·6
H₂O is air and light sensitive and should be stored in a desiccator (if it clumps together,
purchase new).

A.5.2. Reaction

Place ω-hexadecenyl thioacetate (1.68 mmol, **500 mg**), HSiCl₃ (about 5 mL), and
10 to 20 µL of the 4% solution of H₂PtCl₆·6 H₂O in isopropanol into the pressure tube,
seal, and heat at 60 °C in an oil bath overnight. (Figure A.16)

Monitor progress of reaction by NMR. If not complete after 24 hours, add more
catalyst (4% solution of H₂PtCl₆·6 H₂O in isopropanol) and repeat heating for another 24
hours.

**Cool tube in ice each time before opening.** When the reaction is complete, transfer solution to a 50 mL round-bottomed flask. **Remove about 20% of the excess**
Figure A.16. Hydrosilation Reaction
HSiCl₃ using a stream of N₂ (optional). Rotovap to remove rest of HSiCl₃. Grease joints well on rotovap and start up slowly, since HSiCl₃ is easily evaporated. Fill a balloon with N₂ and place over the air intake when releasing the vacuum on the rotovap.

**A.5.3. Purification**

Isolate product using Kugelrohr distillation. See Figure A.10 for set-up, (do not need ice bath). **Grease joints well.** Heat to 100-110°C to remove excess reactant. (white film on flask, some liquid), change collection flasks, then heat to between 120-130°C to obtain product. Use a balloon of N₂ over the air intake when releasing the vacuum. Thioacetate silane is brownish-yellow in color, while the acetate is colorless to pale yellow.

Obtain NMR spectra and compare to Figures A.17 (thioacetate) and A.18 (acetate).
Figure A.17. $^1\text{H}$-NMR Spectrum of 1-(thioacetato)-16-(trichlorosilyl)hexadecane
Peak assignments: 1.20 - 1.65 ppm (m, 30 H), 2.32 ppm (s, 3 H), 2.88 ppm (t, 2 H), 7.25 (solvent, CDCl$_3$).
Figure A.18. $^1$H-NMR Spectrum of 1-(acetato)-11-(trichlorosilyl)undecane
Peak assignments: 1.20 - 1.70 ppm (m, 20 H), 2.05 ppm (s, 3 H), 4.05 ppm (t, 2 H), 7.25 ppm (solvent, CDCl$_3$).
A.6. Self-Assembled Monolayer Formation

A.6.1. Preparation

Distill dicyclohexyl under vacuum at 70-90 °C.

A.6.2. Reaction

Add 80-100 µL of silane to 80-100 mL of dicyclohexyl. Mix solution. Deposition time is 4 to 5 hours for thioacetate, 1 to 2 hours for acetate. After deposition, remove substrate from silane solution and sonicate in CHCl₃ for 5 minutes, then in EtOH for 7 minutes, followed by D.I. H₂O for 10-15 minutes. If a less concentrated solution is used, increase deposition time accordingly. (Example: 40µL of silane in 80 mL of dicyclohexyl, deposit for 8 hours.)

For mixed monolayer deposition, add 50µL of silane total to 80 mL of dicyclohexyl. (Example: for a 50% solution, add 25µL of thioacetate and 25µL of acetate.) Deposition time is 4 to 5 hours. Sonicate in the solvent series described above.

A.6.3. Characterization

Obtain IR spectrum to determine monolayer presence. The thioacetate vibration is at 1697-1692 cm⁻¹, and the acetate vibration is at 1743-1741 cm⁻¹. Compare to Figures A.19 and A.20, respectively. Check film thickness by ellipsometry (on silicon wafer). C₁₆ chains, d = 24 Å ± 4 Å. C₁₁ chains, d = 16 Å ± 4 Å. Use water contact angle to characterize surface. Thioacetate (100%), C.A. = 70° ± 2°; acetate (100%), C.A. = 58° ± 3°.
Figure A.19. FTIR-ATR spectrum for C-16 thioacetate-terminated silane SAM deposited on a silicon ATR crystal. Note: thioacetate carbonyl stretch at 1693 cm⁻¹.
Figure A.20. FTIR-ATR spectrum for C-11 acetate-terminated silane SAM deposited on a silicon ATR crystal. Note: acetate carbonyl stretch at 1742 cm\(^{-1}\).
A.7. Reduction of Thioacetate Silane SAM

A.7.1. Reaction

Add LiAlH₄ to anhydrous ether until bubbling quits (about 1 μg LiAlH₄/200 mL ether). Let sit overnight to settle excess LiAlH₄. Pipet off clear supernatent. Dip substrates into clear solution for a minute. (Can sonicate solution for 30-60 seconds.) Remove and rinse in a 4% HCl solution. Blow dry with N₂. Repeat process 2-3 times.

A.7.2. Characterization

Obtain IR spectrum to observe decrease in thioacetate (and/or acetate) vibrational band. Check film thickness by ellipsometry (on silicon wafer). C-16 chains, d = 20 Å ± 5 Å. C-11 chains, d = 16 Å ± 3 Å. Use water contact angle to characterize surface. Thiol (100%), C.A. = 69° ± 1°; hydroxyl (100%), C.A. = 49° ± 3°.
APPENDIX B
SYNTHETIC ROUTE FOR ACETATE TRICHLOROSILANES

B.1 Synthesis of \(\omega\)-undecenyl acetate

B.1.1. Preparation

Distill \(\text{N(Et)}_3\) (refer to thiol synthetic route, step 1); dry 2- or 3-neck, 50-mL flask, \(\text{N}_2\) inlet and pressure-equalizing addition funnel in oven. Set-up as shown in Figure A.2.

B.1.2. Reaction

Combine 3.5 mL (0.0187 mol) \(\omega\)-undecenyl alcohol and 3.9 mL (0.0281 mol) \(\text{N(Et)}_3\) in flask. Start \(\text{N}_2\) flow and stir. Cool in an ice bath to 0 °C. Combine 50 mL \(\text{CH}_2\text{CH}_2\) and 1.3 mL (0.0224 mol) acetyl chloride and place in addition funnel. Allow the solution to drip slowly into the flask over a period of 30 minutes, while stirring. Stir the solution for another 1.5 to 2 hours once addition is complete. Check product by TLC using 5% ethyl acetate:hexane.

B.1.3. Purification

Purify by flash chromatography if necessary.

B.2. Synthesis of 1-acetato-11-(trichlorosilane)undecane

Refer to Appendix A, Section A.5

B.3. Self-Assembled Monolayer Formation

Refer to Appendix A, Section A.6

B.4. Reduction of Acetate Silane SAM

Refer to Appendix A, Section A.7
APPENDIX C

THEORY AND FABRICATION OF IOW WAVEGUIDES

C.1. IOW THEORY

A planar integrated optical waveguide (IOW), consists of a glass substrate which supports a thin (< 3 μm) dielectric film. (Figure C.1) Light propagates in this film via total internal reflection producing a high reflection density, (typically > 1000 reflections per cm), in a single mode or a few discrete modes (reflection angles). Light can only be coupled into the waveguide at a single or a few selected angles since most of the reflected rays interfere destructively. Total internal reflection was described in section 2.5.1 in conjunction with ATR-FTIR, which uses a thick (1-2 mm) IRE. The effective pathlength of an IOW is increased 1000-fold over a thick IRE, as illustrated in Figure C.2, which allows detection of submonolayer absorbing species.

It has been shown that a ray optics approximation can be used to model the light-guiding behavior of an IOW [119]. Using a ray optics approach, the reflection density, \( N/D \), (defined as the number of internal reflections per unit distance of beam propagation along the IRE/solution interface), is given by

\[
N/D = (2d_2 \tan \theta_i + \Delta_{21} + \Delta_{23})^{-1}
\]

C.1

where \( d_2 \) is the thickness of the IOW, \( \theta_i \) is the mode angle, and \( \Delta_{21} \) and \( \Delta_{23} \) are the Goos-Hanchen shifts at the IOW/solution and IOW/substrate interfaces, respectively. The Goos-Hanchen shifts can be calculated for a step-index, asymmetric planar IOW as described in reference 118. The thickness, \( d_2 \), and refractive index, \( n_1 \), of the waveguide can be determined by measuring the incoupling angles of the mode (or modes), with respect to the stationary laser beam, in TE and TM polarizations, and iteratively solving the eigenvalue for mode propagation in a step index film [119].
Figure C.1. Attenuated Total Reflection Spectrometry of a Thin, Substrate Supported Film. ($\theta_i$ is the angle of propagation, $d_f$ is the film thickness, $I_{in}$ is the intensity of the incident beam, and $I_{out}$ is the intensity of the reflected beam, figure not drawn to scale).
Figure C.2. Comparison Between Conventional and Integrated Optical Waveguides

Conventional (mm thick) Planar Waveguide

Planar Integrated Optical Waveguide (μm thick)

- a very thin internal reflection element (on the order of the propagating wavelength).
- supports approximately 1000 reflections/cm.
- sensitivity allows for ATR measurement on weakly absorbing, submonolayer films.
C.2. WAVEGUIDE FABRICATION

Sol-gel waveguide fabrication has been described in detail by Yang et al., [86]. The general procedure used to fabricate sol-gel waveguides used for the studies described in this document is as follows. A sol-gel solution was prepared by sequentially adding 150 mL of MeSi(OEt)$_3$, (Aldrich), 200 mL of absolute EtOH, (200 Proof, Quantum), and 75 mL of Ti(0Bu)$_4$, (Aldrich), to a clean, dry jar and mixing. SiCl$_4$ (12.5 mL, Aldrich) was added by syringe, bubbling it into the solution to avoid reaction with air. The solution was mixed again, covered, and allowed to cool before use.

Soda lime glass slides were cleaned by soaking in a dilute PCC-54 solution (15 drops detergent/ 100 mL D.I. H$_2$O) for 5 min, followed by scrubbing lightly with cotton or gloved fingers, then sonicating in a fresh PCC-54 solution for 4 min. After rinsing with D.I. H$_2$O, the slides were sonicated in EtOH (reagent grade) for 4 min, rinsed in more EtOH, and dried at 200 °C for 1 hour, then allowed to cool before use.

Clean slides were dip-coated into the sol-gel solution in a clean box in a laminar flow hood with 35-40% relative humidity and temperature between 18-23 °C. Coated slides were immediately transferred to a furnace at about 500 °C for 10-20 minutes, then cooled for 1-2 minutes at the edge of the furnace (to avoid cracking).
APPENDIX D

LOW-ATR ABSORBANCE LINEAR DICHROISM/TIRF ANISOTROPY
THEORY AND CALCULATION OF EXPERIMENTAL VALUES

D.1. THEORY

The LOW-ATR + TIRF method for determination of molecular orientation distribution was developed in this lab. Absorbance linear dichroism (in an LOW-ATR geometry) and TIRF anisotropy methodologies had been each developed separately. The combination theory presented here will focus on the determination of molecular orientation distribution for a circular dipole, since the studies presented in this dissertation make use of a metal-porphyrin (heme) as the spectroscopic probe.

Consider a laboratory coordinate system as illustrated in Figure D.1, (geometry and notation adopted from Thompson et al. [44] and Fraaije et al. [14]). The x-y plane describes the interface between the internal reflection element (IRE) and the protein film (having a lower refractive index) such that light propagating in the IRE is totally internally reflected. When the light propagating in the IRE is polarized (either in TE, transverse electric, or TM, transverse magnetic) then the evanescent field created at each reflection, which decays exponentially along the z-axis, is also polarized. The electric field for TM polarized light projects mainly along the z-axis and with a small x-axis component, while the electric field for TE polarized light projects only along the y-axis. Spectroscopic probes (the heme groups) in the film on the IRE surface absorb light from the evanescent wave.

In the laboratory coordinate system, the heme plane is designated as the x'-z' plane. (Figure D.1) Its position relative to the laboratory coordinates can be described by $\theta$, the angle between the heme plane and the z-axis (or by $\beta$, the angle between the normal to the
Figure D.1. Heme Coordinate System. (x,z axes are in the lab coordinate system, x'-z' is the plane of the porphyrin, $\theta$ is the angle between the heme plane and the z-axis, $\beta$ is the angle between the normal to the heme plane and the z-axis, $\phi$ is the angle between the x' and x axes, $\alpha$ is the angle between the absorption dipole $\mu$, and the z'-axis).
heme plane and the z-axis, with $\beta + \theta = 90^\circ$). In-plane positioning is described by $\phi$, the angle between the $x'$ and $x$ axes and $\alpha$, the angle between the absorption dipole $\mu_1$ and the $z'$-axis. Polarized absorption studies of heme protein crystals (references 20-22 in [41]) show that the heme absorption bands in the visible range are $x,y$ polarized in the molecular plane. Therefore, it can be approximated that $\mu_1 = \mu_2$ and the absorption intensity is not a function of $\alpha$ (circularly polarized). Another approximation can be made that there is not preferential molecular orientation in the $x'$-$z'$ plane, such that all $\phi$ values are equivalent [41]. This leaves the mean tilt angle ($\theta$) to describe molecular orientation. Orientation in the $x$-$z$ plane can be determined using polarized radiation. A planar heme group oriented with its absorbance dipoles parallel to the IRE surface ($x$-$y$ plane), will absorb only light polarized in the $x$-$y$ plane, (TE), while a planar heme group perpendicular to the surface will primarily absorb light polarized along the $z$-axis, (TM), with some absorbance in the $x$-$y$ plane, (TE). Orientation of the heme group at an angle, $\theta$, from the surface normal will yield light absorbance from both polarizations, the ratio of which is related to its specific position according to the following equation:

$$\rho = \frac{A_{TE, Corr}N_{TM}}{A_{TM, Corr}N_{TE}} = \frac{\frac{1}{2}E_2^2 \int_0^{\pi/2} N(\beta)(1 + \cos^2 \beta) \sin \beta d\beta}{\frac{1}{2}E_2^2 \int_0^{\pi/2} N(\beta)(1 + \cos^2 \beta) \sin \beta d\beta + E_2^2 \int_0^{\pi/2} N(\beta) \sin^2 \beta \sin \beta d\beta}$$  \hspace{1cm} (D.1.1)

where $\rho$ is the dichroic ratio, $A_{TE, Corr}$ and $A_{TM, Corr}$ are the corrected absorbances of TE and TM polarized light by the film, $N_{TM}$ and $N_{TE}$ are the number of internal reflections over which the measurement is made in each polarization, $E_2^2$, $E_x^2$, and $E_z^2$ are the squared electric field amplitudes of the evanescent wave as calculated by the equations presented in section D.3, and $N(\beta)$ is the orientation distribution. For these measurements, $N(\beta)$ has been chosen to be represented as a Gaussian function:
\[ N(\beta) = \exp \left[ -\frac{(\beta - \beta_\mu)^2}{2\beta_\sigma^2} \right] \]

where \( \beta_\mu \) is the mean tilt angle and \( \beta_\sigma \) is the standard deviation about the mean.

Since \( N(\beta) \) is a function of two variables, two experimental measurements are needed. Absorbance linear dichroism, in an attenuated total reflection geometry using an integrated optical waveguide, provides one measurement, while a steady state anisotropy experiment, using total internal reflectance fluorescence (TIRF), provides a second. Figure D.2 shows the heme plane along the normal axis, where \( \mu_1 \) and \( \mu_2 \) are perpendicular to each other in the heme plane. Two emission dipoles are also present, \( v_1 \) and \( v_2 \), which are also perpendicular to each other in the plane of the heme. They are at an angle \( \gamma \) to the absorbance dipoles, where \( \gamma \) depends on the extent of emission depolarization in the heme plane. For a metallophyrin with \( D_{4h} \) symmetry, the emission dipoles, as with the absorbance dipoles, can be considered circularly polarized such that \( \gamma = 45^\circ \), (randomized emission in the plane). Side chains and the heme environment may affect the heme symmetry, thereby decreasing the value of \( \gamma \).

As with absorbance linear dichroism measurements, in TIRF, the extent of absorbance by the heme group of each light polarization is dependent upon the orientation of the heme relative to the surface normal. Subsequent fluorescence emission is also polarized and the emission intensity is proportional to the absorbance in each polarization. Therefore the steady state anisotropy can be calculated using:

\[ r = \frac{I_z - I_y}{I_z + 2I_y} \]

where \( I_z \) and \( I_y \) are the emission intensities for polarized excitation along the \( z \)- and \( y \)-axes, respectively. To simplify the relationship between the emission intensities and molecular orientation, several assumptions must be made. First, it is assumed that the approximations described above for simplification of the absorbance measurements hold.
Figure D.2. Schematic of the Heme Plane Showing the Direction of the Absorption and Emission Dipoles Relative to Each Other.
true; second, a low numerical aperture objective is used to collect emission so that its intensity is proportional to the projection of the emission dipole on the x-y detection plane; third, there is no polarization bias in the detection system itself; and fourth, the excited state lifetime is short enough that essentially no depolarization occurs due to molecular motion or intermolecular energy transfer. With these assumptions, the intensities can be related to orientation as follows:

$$I_z = E_z^2 \frac{\int_0^\pi N(\beta) \sin^2 \beta (1 + \cos^2 \beta) \sin \beta d\beta}{\int_0^\pi N(\beta) \sin \beta d\beta}$$

$$I_y = E_y^2 \frac{\int_0^\pi N(\beta) (1 + \cos^2 \beta)^2 \sin \beta d\beta}{\int_0^\pi N(\beta) \sin \beta d\beta}$$

where $E_z^2$ and $E_y^2$ are the squared electric field amplitudes of the evanescent wave along the z- and y-axes at the origin, (given in section D.3). Since the normal to the heme plane is the major axis of symmetry for the porphyrin, integration must be about $\beta$ to provide orientation information. The measurement of both $\rho$ and $r$ for a system allows determination of $\beta_n$ and $\beta_o$ by simultaneously solving equations D.1.1 and D.1.3.

**D.2: CALCULATION AND CORRECTION OF EXPERIMENTAL VALUES**

The calculations of $\rho$ and $r$ from experimental measurements require some corrections before the values can be used to solve for $\beta_n$ and $\beta_o$. The experimental values obtained for calculation of the dichroic ratio, $\rho$, consist of measurements of the light intensity as a function of distance of propagation of the evanescent wave. The slope of the
log of the intensity versus distance, the loss coefficient, is proportional to the absorbance. The actual absorbance does not need to be calculated because $\rho$ is a ratio of the absorbances in each polarization, which is equivalent to the ratio of the loss coefficients in each polarization. The loss coefficients for the film, $A_{h,TE}$ and $A_{h,TM}$ are corrected for loss due to the substrate by subtracting off the loss coefficients obtained with only buffer in the flowcell, $A_{h,TE}$ and $A_{h,TM}$.

The value of $A_{x,TM}$ is a combination of absorbance of light along the z- and x-axes. In order to ratio absorbances for pure TM to pure TE, the absorbance measurement in TM must be corrected for the x-axis component. This is accomplished by subtracting off the amount due to absorbance from the x-axis component, where the square of the electric field intensity in the x-axis is considered equivalent to that in the y-axis, since the absorbance dipoles are assumed to be isotropic in the x-y plane:

$$A_{TM,Corr} = \frac{A_{TM} \cdot \left( \frac{N_{TM} E_z^2}{N_{TE} E_y^2} \right) A_{TE}}{E_z^2 N_{TM}}$$ \hspace{1cm} D.2.1

$$A_{TE} = \frac{A_{TE}}{E_y^2 N_{TE}} \hspace{1cm} D.2.2$$

This same correction must be done for the measured fluorescence intensities as well:

$$I_z = \frac{[I_{TM} - (I_{TE} \cdot \frac{E_z^2}{E_y^2})]}{E_z^2} \hspace{1cm} D.2.3$$

$$I_y = \frac{I_{TE}}{E_y^2} \hspace{1cm} D.2.4$$

The corrected values are then substituted into equations D.1.1 and D.1.3, respectively.
D.3. CALCULATION OF ELECTRIC FIELD AMPLITUDES

The following equations are used to calculate the electric field amplitudes along each axis:

\[ E_x = \frac{2(\sin^2 \beta_{TM} - n_{21}^2)^{1/2} \cos \beta_{TM}}{(1 - n_{21}^2)^{1/2} [(1 + n_{21}^2)\sin^2 \beta_{TM} - n_{21}^2]^{1/2}} \]  
\[ E_y = \frac{2 \cos \beta_{TE}}{(1 - n_{21}^2)^{1/2}} \]  
\[ E_z = \frac{2 \sin^2 \beta_{TM} \cos \beta_{TM}}{(1 - n_{21}^2)^{1/2} [(1 + n_{21}^2)\sin^2 \beta_{TM} - n_{21}^2]^{1/2}} \]

Where \( \beta_{TM} \) and \( \beta_{TE} \) are the angles of total internal reflection in mode 0 for each polarization, respectively, and \( n_{21} \) is the index of refraction for the waveguide.
APPENDIX E

LANGMUIR ISOTHERM FITS FOR ADSORPTION ISOTHERMS

Figure E.1. Plots of Langmuir Isotherm Fits for Wild Type Yeast Cyt c on Thiol SAM Surfaces. Calculated curves (solid lines) were obtained using the binding affinities determined using an iterative Origin 4.1 fitting program. Measured values are shown as black squares.

Yeast Cyt c on Thiol SAM Surface - Low Concentration Region

Yeast Cyt c on Thiol SAM Surface - High Concentration Region
Figure E.2. Plots of Langmuir Isotherm Fits for Wild Type Yeast Cyt c on Hydroxyl SAM Surfaces. Calculated curves (solid lines) were obtained using the binding affinities determined using an iterative Origin 4.1 fitting program. Measured values are shown as black squares.
Figure E.3. Plots of Langmuir Isotherm Fits for Wild Type Yeast Cyt c on Mixed SAM Surfaces. Calculated curves (solid lines) were obtained using the binding affinities determined using an iterative Origin 4.1 fitting program. Measured values are shown as black squares.

Yeast Cyt c on Mixed SAM Surface - Low Concentration Region

Yeast Cyt c on Mixed SAM Surface - High Concentration Region
Figure E.4. Plots of Langmuir Isotherm Fits for Horse Heart Cyt c on Thiol SAM Surfaces. Calculated curves (solid lines) were obtained using the binding affinities determined using an iterative Origin 4.1 fitting program. Measured values are shown as black squares.
Figure E.5. Plots of Langmuir Isotherm Fits for Horse Heart Cyt c on Hydroxyl SAM Surfaces. Calculated curves (solid lines) were obtained using the binding affinities determined using an iterative Origin 4.1 fitting program. Measured values are shown as black squares.
Figure E.6. Plots of Langmuir Isotherm Fits for Horse Heart Cyt c on Mixed SAM Surfaces. Calculated curves (solid lines) were obtained using the binding affinities determined using an iterative Origin 4.1 fitting program. Measured values are shown as black squares.
Figure E.7. Plots of Langmuir Isotherm Fits for Variant Yeast Cyt c on Thiol SAM Surfaces. Calculated curves (solid lines) were obtained using the binding affinities determined using an iterative Origin 4.1 fitting program. Measured values are shown as black squares.
Figure E.8. Plots of Langmuir Isotherm Fits for Variant Yeast Cyt c on Hydroxyl SAM Surfaces. Calculated curves (solid lines) were obtained using the binding affinities determined using an iterative Origin 4.1 fitting program. Measured values are shown as black squares.

**Variant Yeast Cyt c on Hydroxyl Surface - Low Concentration Region**

- Monolayer vs. Solution Concentration (μM)
- Calculated and Measured values

**Variant Yeast Cyt c on Hydroxyl SAM Surface - High Concentration Region**

- Monolayer vs. Solution Concentration (μM)
- Calculated and Measured values
Figure E.9. Plots of Langmuir Isotherm Fits for Variant Yeast Cyt c on Mixed SAM Surfaces. Calculated curves (solid lines) were obtained using the binding affinities determined using an iterative Origin 4.1 fitting program. Measured values are shown as black squares.

Variant Yeast Cyt c on Mixed SAM Surface - Low Concentration Region

Solution Concentration (μM)

Variant Yeast Cyt c on Mixed SAM Surface - High Concentration Region

Solution Concentration (μM)
REFERENCES


