BIOANALYTICAL APPLICATIONS OF CHEMICALLY MODIFIED SURFACES

By

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A Dissertation

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Doctor of Philosophy

in

Chemistry

December 2009

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ABSTRACT

The design and development of chemically modified surfaces for bioanalytical applications is presented. Chemical surface modification is demonstrated to be a method to control surface properties on the molecular level by selecting the appropriate substrate, linking chemistry, and terminal group functionality. These systems utilize spontaneous interactions between individual molecules that allow them to self-assemble into larger, supramolecular constructs with a predictable structure and a high degree of order. Applications investigated in this thesis include: surface patterning, switchable surface wettability, and biological sensor devices that combine surface based molecular recognition, electrochemical detection methods, and microfluidics.

A multilayered approach to complex surface patterning is described that combines self-assembly, photolabile protecting groups, and multilayered films. A photolabile protecting group has been incorporated into molecular level films that when cleaved leaves a reactive surface site that can be further functionalized. Surface patterns are created by using a photomask and then further functionalizing the irradiated area through covalent coupling. Fluorophores were attached to the deprotected regions, providing visual evidence of surface patterning. This approach is universal to bind moieties containing free amine groups at defined regions across a surface, allowing for the development of films with complex chemical and physico-chemical properties.

Systems with photoswitchable wettability were developed by fabricating multilayered films that include a photoisomerizable moiety, cis-trans-dicarboxystilbene. When this functionality was incorporated into a multilayered film using non-covalent interactions, irradiation with light of the
appropriate wavelength resulted in a conformational change that consequently changed the hydrophobicity of the substrate. Methods were investigated to increase the reversibility of the photoswitching process by creating surface space between the stilbene ligands. Utilizing mixed monolayers for spacing resulted in complete isomerization for one cycle, while the use of SAMs with photolabile groups produced surfaces that underwent isomerization for three complete cycles.

A microfluidic device platform for ion sensing applications has been developed. The platform contains components to deliver small volumes of analyte to a surface based microelectrode array and measure changes in analyte concentration electrochemically in an analogous method to that used in conventional electrochemical cells. Crown ether derivatives that bind alkali metal ions have been synthesized and tested as ionophores for a multi-analyte device of this type, and the sensing platform was demonstrated to measure physiological relevant concentrations of potassium ions. Advantages of this design include: high sensitivity (μM to mM), small sample volumes (less than 0.1 mL), multi-analyte capabilities (multiple working electrodes), continuous monitoring (a flow through system), and the ability to be calibrated (the system is reusable).

The self-assembled systems described here are platform technologies that can be combined and used in molecular level devices. Current and future work includes: photopatterning of gold and glass substrates for directed cell adhesion and growth, the design and synthesis of selective ion sensors for biological samples, multi-analyte detection in microfluidic devices, and incorporating optical as well as electrochemical transduction methods into sensor devices to allow for greater sensitivity and self-calibration.
ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor, Professor Grant McGimpsey for the opportunity to work in his lab and for always encouraging and challenging me in my research. It has been an exciting adventure and a tremendous learning experience, and I am grateful for the opportunity.

I would also like to sincerely thank Professor Chris Lambert who has acted as my co-advisor for most of my graduate career. Professor Lambert encouraged me to attend graduate school and his patience and availability has made my time here more productive and rewarding than I thought possible. He has always challenged me to be a better thinker, writer, and scientist and I would not be in the position I am in today without his support, dedication, and commitment to my success.

To my committee, Professor John MacDonald and Professor James Dittami, many thanks for all of their help during my graduate career and for taking the time to read my dissertation, their assistance has been much appreciated. I would also like to thank my external examiner, Professor Robert Redmond for taking the time out of his schedule to be at my thesis defense.

I would like to thank current and former members of our lab group for their assistance, encouragement, and collaboration with my research. Particularity, I would like to acknowledge Ms. Morgan Stanton, Mr. Eugene Douglass, Dr. Nantanit Wanichacheva, Dr. Chris Cooper, Dr. John Benco, Dr. Hubert Nienaber, and Dr. Ernesto Soto for all of their help. I would especially like to thank Mr. Eftim Milkani who has worked alongside me for the past several
years and has contributed to many of the projects presented here. Eftim is an excellent scientist and has always been willing to take the time to discuss a problem, share ideas, or listen to me complain, and my time at WPI would not have been as successful or productive without his help.

I would like to thank all the faculty, staff, and graduate students in the Chemistry and Biochemistry department, particularly Professor Kristin Wobbe, Professor James Pavlik, Professor Robert Connors, Dr. Ilie Fishtik, Ms. Paula Moravek, and Ms. Ann Mondor for all of their help. I would like to acknowledge Mr. Jack Ferraro and Mr. Douglas White for their invaluable assistance in fabricating many of the devices and equipment used in my research. Also, many thanks to other members of the WPI community, especially Professor George Pins, Professor Ray Page, Professor Tanja Dominko, Professor Nancy Burnham, Mr. Dave Messier, and Ms. Elizabeth Stepien for their assistance. I would also like to thank Dr. Dan Wayner of the NRC in Canada for his helpful discussions of portions of my research and for giving me the opportunity to spend some time in his lab and learn several new techniques.

To my friends at Gateway, especially Dr. Katie Bush, Mr. Jacques Guyette, Ms. Angie Throm, Ms. Tracey Gwyther, and Mr. Jeremy Skorinko, thanks for their support and assistance. Whether it was through a game of softball, a few cold beers, or a conversation over lunch, they have helped me keep my sanity and perspective during a challenging time, and I am honored to be considered one of their friends.

Thanks to my family and friends for always being there for me; especially to my parents, Jean and Fred, my sister Libby, my brother-in-law Shaun, my uncle Rob, and my grandmother Narnie
for their constant encouragement during my graduate career. They have always supported all of my endeavors, whether personal or professional, and my success is very much a testament to them. I would also like to thank Mr. Eliot Pitney, Mr. Josh Cohen, Mr. Peter Nash, and Mr. Kent Geisel for being some of the best friends that anyone could ever hope for.

Most of all, to my wife, best friend, and partner in life Arielle for all of her support, encouragement, and love over the past few years. She has made me a far better person and I am incredibly fortunate to be able to share my life with her. Arielle’s patience and understanding during my time in graduate school has been far greater than I could have ever hoped for and this thesis is dedicated to her.

And, of course, my best buddy Wally, who is always happy to see me no matter the time of day or type of mood I’m in.
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1: INTRODUCTION
Nanotechnology, the study of molecular scale systems, is a research field with applications that range from energy production and storage, to new types of electronic devices, to healthcare.\textsuperscript{1-2} Focusing on materials and structures with dimensions of 100 nm or smaller, nanotechnology involves studying individual atoms and molecules as well as the ordered, macromolecular assemblies that they form. One area of nanotechnology that has been examined in the past several decades is molecular level surface functionalization.\textsuperscript{3-4} Chemical surface modification, where a chemical bond is formed between a substrate and the functionalizing unit, can be used to control surface properties and provide surface functionality that the substrate alone does not possess, such as: degree of wettability, biocompatibility, absorptivity, and molecular recognition.\textsuperscript{5}

Self-assembly describes an entropically driven process in which a disorganized system of individual units organizes into an ordered arrangement through interactions between the individual components, without external influence.\textsuperscript{6} Hydrogen bonding, $\pi-\pi$ stacking, and van der Waals forces are types of interactions that drive self-assembly. Overall, the free energy of the system decreases as the units organize; the macromolecular assembly is more thermodynamically stable than the unassembled components.

Self-assembled monolayers (SAMs) are an established method of functionalizing surfaces through selection of appropriate attachment chemistry.\textsuperscript{4-5,7-8} Some of the first studies of molecular, organic films on surfaces were performed by Irving Langmuir and Katherine Blodgett in the 1930s, who developed methods to transfer layers formed at the air/water interface onto solid supports (referred to as Langmuir-Blogett (LB) films).\textsuperscript{9} LB films are formed by
compressing amphiplilic molecules on the surface of a liquid into an ordered arrangement, and transferring the resulting film to a solid substrate. Although the LB technique generates a molecular film, physisorption interactions (rather than formation of a covalent bond) and the complicated deposition process limits applicability.

Studies by Zisman, et al. in the 1940s demonstrated that hydrophilic platinum substrates immersed into solutions of polar organic molecules in non-polar solvents resulted in hydrophobic surfaces, which was attributed to the formation of oriented monolayers. In 1983 Lucy Netzer and Jacob Sagiv reported on the absorption of silane surfactants on surfaces to generate oriented monolayers and multilayer films. Their work was described in New Scientist; the films they fabricated were called “self-assembling monolayers,” the first use of the term. Also in 1983, Nuzzo and Allara discovered the absorption of organic disulfide monolayers on gold, and since their initial report thiols on gold have likely become the most studied SAM system. Currently, there are over 15,000 scientific papers that discuss self-assembled monolayers.

A diagram of a SAM that shows the three primary molecular components; linking group (orange), backbone chain (black), and terminal group (R) is shown in Figure 1.1. The linking group provides the attachment chemistry; linking groups include thiols for gold, silver, platinum, palladium, copper, mercury, and stainless steel substrates and trichloro-, trimethoxy-, or triethoxy- silanes for silica (e.g.; glass, quartz, silicon) or metal oxide (e.g.; indium tin oxide, ITO) surfaces. The backbone chain, usually comprised of alkyl (CH\_2) groups, organizes and stabilizes the film due to inter-chain (van der Waals) interactions. Terminal groups include methyl (CH\_3), carboxylic acid (COOH), hydroxyl (OH), amine (NH\_2),
and other chemistries that control the properties (wettability, etc., discussed above) of the functionalized surface.

![Diagram of a SAM showing substrate (grey) linking group (orange), backbone chain (black), and terminal group (R).](image)

**Figure 1.1:** Diagram of a SAM showing substrate (grey) linking group (orange), backbone chain (black), and terminal group (R).

Two specific examples of SAMs are shown in Figure 1.2, dodecanethiol on gold and dodecyltrimethoxysilane on glass. These 2D representations are not meant to depict the molecular surface arrangements; however, differences between these systems are noted. Thiols on gold are dynamic; individual molecules translate across the surface and organize into an arrangement that maximizes chain-chain interactions. Silanes on glass (or other silica and metal oxide substrates) form a polysiloxane network (shown in Figure 1.2) that is not dynamic. Alkanethiols on have a sulfur-sulfur separation of ~ 5 Å and organize into a tilted arrangement.
with a cant angle of approximately $30^\circ$ to the surface normal. Polysiloxane surface networks have an inter-chain spacing of $\sim 4.4$ Å and orient nearly perpendicular to the substrate with cant angles of $0^\circ$ to $5^\circ$ reported.

Monolayers of alkanethiols deposited on gold are one of the most studied self-assembled surface systems due to specific advantages of the substrate, the thiol group, and the interaction between the two. Gold does not easily oxidize, does not react with most chemicals, is electrically conductive, and can be deposited by thermal evaporation as a thin film on solid supports such as glass or silicon. SAMs can be also formed on polycrystalline bulk gold surfaces that are polished or cleaved. Thin films on gold can be characterized by methods that include physical (contact angle (CA)), electrochemical (cyclic voltammetry (CV)), impedance

Figure 1.2: SAMs of dodecanethiol on gold (left) and dodecytrimethoxysilane on glass (right).
spectroscopy (EIS$^{37}$), spectroscopic (optical ellipsometry,$^{38}$ infrared spectroscopy (IR)$^{39}$, surface plasmon resonance spectroscopy (SPR)$^{40}$, X-ray photoelectron spectroscopy (XPS)$^{41}$), and microscopic (atomic force microscopy, (AFM)$^{42}$ scanning tunneling microscopy(STM)$^{43}$). Gold is compatible with biological systems and modified substrates can be used as templates for studies such as cell adhesion.$^{44}$

Alkanethiols are stable compounds and synthetic procedures to incorporate thiol moieties into molecules are well established.$^{16}$ Thiols have a high affinity for gold substrates, and displace adventitious surface contaminants. SAMs can be prepared under ambient laboratory conditions; manipulation of both gold substrates and thiol compounds does not require sophisticated equipment such as clean rooms or ultra-high vacuum. Monolayers utilizing thiol-gold interactions are stable to conditions including immersion organic solvents and aqueous solutions.$^{45-47}$ SAMs of alkanethiols on gold have been used for applications including: photovoltaics,$^{48}$ cell adhesion,$^{49}$ protein adhesion,$^{50}$ pH sensing,$^{51}$ glucose sensing,$^{52}$ and ion sensing.$^{53}$

Monolayer preparation involves cleaning the gold substrate using an oxidizing acid or oxygen plasma, immersion in a dilute (~ 1 mM) thiol solution in an organic solvent, incubation of the substrate in the adsorbate solution to allow for SAM organization (12-24 hours), and withdrawal and rinsing of the surface to remove any physisorbed material. The mechanism of SAM formation involves a fast (seconds to minutes) interaction between the thiol group and the metal surface, followed by a slow (minutes to hours) reorganization of the film due the interatomic forces between the molecules that make up the monolayer.$^{3-4}$ Compounds with alkyl chains of
10 to 18 carbons in length are used to maximize inter chain van der Walls forces that organize and stabilize the film.

The nature of the thiol-gold bond and the process by which ordered monolayers form remains controversial and not precisely understood.\textsuperscript{3} The reaction is generally described as an oxidative addition of the thiol to the gold surface followed by reductive elimination of hydrogen as \( \text{H}_2 \) (or the oxidative conversion to water in the presence of oxygen). This results in the formation of a covalent Au-S bind with a bind strength estimated to be \( \sim 40 \text{ kcal/mol} \).\textsuperscript{4} The interaction between thiol compounds and gold substrates has been studied by scanning tunneling microscopy (STM) and has been described as a multi-step process.\textsuperscript{16}

At low surface concentrations (immediately following substrate immersion) thiol molecules chemisorb to the gold and form what are referred to as striped phases in which individual thiol molecules lay flat on the substrate and rapidly transverse across the surface.\textsuperscript{16} As the surface concentration of thiols increase, nucleation islands from where neighboring molecules meet as they move across the surface. At these nucleation islands the film begins to organize as the alkyl chains cease lying flat and orient away from the surface due to interchain interactions. As the initial thiol molecules orient themselves into growth islands additional surface space is created as the flat striped phases form vertical arrangements with respect to the substrate. This results in thiol molecules from solution to bind to the substrate and fill in any gaps as the film continues to organize.

Although the thiol-gold interaction is strong, thiols on gold have been shown to be dynamic in
that they can migrate across the substrate in solution (by STM studies), although the dynamic nature of alkanethiols on gold substrates is not well understood.\textsuperscript{4} Generally accepted is that thiols molecules bind at the three-fold hollow sites of a Au(111) lattice (Au(111) is thermodynamically most favorable for evaporated gold films) with a separation of 5 Å as previously mentioned. Migration of thiolates between neighboring hollow sites likely proceeds by movement of the thiol to a top site (directly above a single gold atom), however whether the migration mechanism involves desorption and reformation of the gold thiol bond or movement of the entire Au-S molecular assembly has not been determined.\textsuperscript{4} In either case the final organization of the film is driven thermodynamically by the orientation of the backbone alkyl chains of the alkanethiol molecules.

Supramolecular surface bound architectures, such as those described above, are used to add properties to a substrate in a controlled manner, without sacrificing the useful properties of the initial surface (e.g., rigidness, transparency, electrical conductivity). For example; a hydrophilic surface can be modified to repel water, a surface that does not absorb visible light can be made to capture electromagnetic radiation, and surfaces can be prepared that will respond to changes such as electric potential, heat, pH, or other stimuli. Surface modification methods use the chemistry of individual atoms and molecules on the nanoscale to affect properties on a significantly larger scale.

An example of a functional surface investigated here is one that can be patterned to introduce different types of functionality in different regions of a surface. In this thesis a patterning method is described which can be used to repeatedly activate and functionalize a substrate with
new types of chemistry in specific regions. A significant limitation to some types of patterning methods (such as microcontact printing where a stamp with a pattern is inked with monolayer solution and applied to a substrate)\textsuperscript{54} is that once the surface is patterned, no further functionalization can take place. Using the patterning method described here the same surface can be repeatedly activated towards further modification in different areas, which is of interest for biological applications that include sensors and cell adhesion.

Surfaces that sense biologically relevant analytes (such as alkali metal ions and blood glucose, described in detail below) are useful as disease indicators.\textsuperscript{55} Particular interest is in designing surfaces and devices that sense multiple analytes (for example K\textsuperscript+ ions along with glucose). In order to fabricate multi-analyte sensors it is necessary to develop methods to attach different sensing moieties to the same substrate. A surface patterning method that uses light to activate a portion of the substrate to modification with one sensor, and then allows for the process to be repeated in another region, will accomplish the goal of generating a multifunctional/multicomponent sensing substrate. The system described uses a monolayer with a photolabile portion that when cleaved, reveals a reactive site. New chemistry is introduced at the reactive site and the process is repeated in other regions of the surface.

Cell adhesion and growth on modified substrates is also of biological interest.\textsuperscript{56-57} Particularly, the controlled growth of nerve cells for repairing damaged neuronal networks is significant. Injured nerve cell networks result in the loss of muscular and sensory function and current treatments, such as nerve grafts, often do not result in the function that was damaged being restored. Substrates that specifically direct the growth of neurons in defined arrangements can
be used for both basic neuroscience research as well as have the potential to be implanted in the
body to reestablish broken neuronal connections. Patterned surfaces fabricated with different
types of chemistries can be used to control neuronal growth in defined arrangements, and the
patterning method developed is designed to allow for incorporation of more than one type of
chemistry (terminal functionality) on the same substrate.

Systems that undergo photoinduced changes in wettability are of interest for microfluidics.
Microfluidic or “lab-on-a-chip” approaches involve scaling down processes to chip formats with
small sample volumes. For example, microfluidic reactors allow for chemical reactions to take
place on a small scale which minimizes the amount of reactants needed and also the amount of
waste produced. These types of systems can be used to run a large number of reactions in a
fraction of the space with a fraction of the supplies that would be necessary for a conventional
bench reaction. Also, by sequestering the reaction to a sealed microfluidic system the individual
running the process is not in contact with harmful reactants or products, improving safety.
Microfluidic sensors allow for analyte to be detected using small (μL) sample volumes, which
are of particular interest for physiological monitoring devices that use a minimal fluid volume.

These types of microfluidic systems require the precise control of fluid flow. A light driven
fluidic gate that operates due to a change in surface wettability between hydrophobic (gate
closed) and hydrophilic (gate open) would be useful in controlling the mixture of liquids in a
microfluidic reactor. A reversible gate would allow specific volumes of liquid to be introduced
to a reaction chamber by applying a stimulus of light to open and then close the fluidic gate,
which is desirable in that it maintains a closed microfluidic system with no moving parts as the
gate is simply a molecular layer on the substrate. Reversible systems that are fabricated from multilayered films are advantageous because the film can be quickly assembled from simple components and also because multilayered films can be formed in specific regions of a substrate using surface patterning.

Surface based sensing systems are investigated with the goal of measuring alkali metal concentrations present in physiological samples. Electrolyte levels (particularly Na\(^+\) and K\(^+\)) in the body are important for nerve and muscular function as well as control of the level of hydration.\(^{62}\) Na\(^+\) and K\(^+\) pass through ion channels in the cell membrane to control neuronal and muscular activity, and if the physiological amounts of these electrolytes are not at the proper levels, muscle weakness or involuntary muscle contractions can occur. Normal physiological concentrations of Na\(^+\) and K\(^+\) are 135-145 mM and 3-5 mM, respectively.\(^{63}\) Severe deviations of these electrolyte levels can lead to both cardiac and neurological problems which can be life threatening. Also, the kidneys excrete excess electrolytes and a high level in the bloodstream is indicative of a problem with renal function. Systems that monitor levels of alkali metal ions, and particularly those designed to measure more than one analyte, are therefore useful for diagnosis and treatment of medical complications.

Monitoring lithium ion concentration in physiological fluids is vital for patients that are being treated with lithium therapy for manic-depressive disorders or hyperthyroidism.\(^{64}\) Although lithium therapy is effective in treating these disorders, there is a very narrow therapeutic range in the bloodstream. The therapeutic range for lithium is on the order of 0.5 to 1.2 mM, however at concentrations above 1.5 mM becomes toxic and at concentrations above 2.0 mM becomes
Therefore patients treated with lithium therapy need to be closely monitored to ensure that lithium levels remain in the desired therapeutic range and do not cross into the dangerous or fatal levels. Sensor platforms that detect lithium, and are capable of continuous monitoring, are therefore of particular interest to patients with these disorders and physicians treating them.

Blood glucose monitoring is needed for individuals with diabetes. Diabetes is a disease in which the level of sugar in the blood cannot be properly controlled due to an insulin deficiency. Insulin, which is produced in the pancreas, controls the ability of cells to uptake glucose and convert it into energy. Without proper insulin control glucose levels in the blood increase which can lead to a series of health issues including vision problems, circulation problems, and organ failure if left untreated. Two main types of diabetes include Type I (where the body does not produce insulin) and Type II (which results when insulin does not properly control glucose uptake by cells). Individuals with diabetes (particularly those with Type I which are treated by insulin injections) require daily monitoring of blood glucose levels in order to determine how much insulin to take and how much sugar to ingest.

Normal blood glucose levels are in the range of 4 to 10 mM depending on when a meal was last eaten as glucose levels typically increase upon ingesting food until the cells of the body break down the sugar into energy. Levels in the range of 15 to 20 mM or above are indicative of high blood sugar, or hyperglycemia, which is a dangerous condition if left untreated. Individuals with Type I diabetes need an immediate insulin injection to lower their blood sugar if a high level is detected. Although devices are currently on the market that monitor blood glucose levels, incorporation of glucose sensing into a multi-analyte device that also monitors other
physiological levels (such as alkali metal ions) will increase the usefulness of the system as a diagnostic aid.

It is emphasized that the sensors used in this work were chosen as a proof-of-principle for the sensor device platform, in that they demonstrate the incorporation of different types of sensing units that respond to different analytes. A particular application and drive behind developing the sensing platform described here is for continuous, remote monitoring. The ability to monitor conditions of individuals who are undertaking strenuous activity (such as soldiers in the field or athletes participating in a sport) from a remote location would allow for medical personnel recognize a medical problem and intervene prior to the problem becoming life threatening. For example, marathon runners (particularly on very hot days) are in danger of water intoxication (hyper-hydration) if they inadvertently consume too much water which causes electrolyte levels in the body to be out of balance and can result in improper brain function leading to death. If electrolyte levels (Na\(^+\) and K\(^+\)) are monitored continuously during strenuous activities such as running or other types of physical training, deviations from normal levels can be detected and treated prior to reaching levels that are life threatening.

The applications described above were investigated by fabricating chemically modified surfaces using self-assembled monolayers formed through thiol gold interactions. These studies use functional surfaces that are assembled using simple molecular components to create supramolecular systems that can be patterned, undergo wettability changes, and respond to analyte binding.
The approach used in our lab to modify surfaces is to: (1) choose and optimize the linking chemistry for the substrate, (2) determine the terminal group (CH₃, COOH, OH, NH₂, etc.) required, (3) synthesize the molecular components used to fabricate the monolayer, (4) deposit and characterize the thin film, and (5) test the system with the application that it was designed for (wettability, sensing, cell adhesion, etc.). This method approaches surface modification by first examining the chemistry of the substrate and then building upon that by using both self-assembling molecular interactions and the ability as chemists to form bonds between atoms and groups of atoms.

An example of this method is in the design of a sensing surface for alkali metal ions. Sensors that use electrochemical detection require a conductive substrate and selection of gold-thiol linking chemistry will satisfy this requirement. A crown ether terminal group provides a metal ion binding site which can be linked to a thiol group through an alkyl chain for surface attachment. Deposition of the monolayer and characterization to determine surface coverage, degree of order, and terminal functionality will confirm the film structure. Finally, testing of the modified substrate in the presence of metal ions using electrochemical detection methods confirms successful fabrication of a surface sensor.

The overall goal of the work presented is to use interactions on the molecular level to effect surface properties for bioanalytical and biomedical device applications. The research undertaken and described here has the following specific aims:

- Create a ‘toolbox’ approach to surface functionalization that can be used to assemble complex systems from simple components.
- Develop a surface patterning method that allows multilayered (and therefore multicomponent) films to be formed in specific regions of a substrate for applications that include multi-analyte sensors as well as scaffolds for cell growth.
- Fabricate films that undergo reversible photo-induced changes in wettability for applications as microfluidic gates.
- Design and implement a microfluidic device that is capable of multi-analyte detection for monitoring multiple physiological samples.

The results described are a platform technology that can be used to control surface properties and the systems developed are not limited to single applications, but applicable to different types of bioanalytical studies.

This dissertation is divided into six chapters. Chapter 2 provides detailed descriptions of the surface characterization techniques used in the studies presented, along with experimental details for these techniques. Chapter 3 provides a brief overview of the surface modification approach (specifically multicomponent films) used for the studies described in the later chapters. Chapters 4-6 discuss surface patterning, switchable wettability, and sensor devices, respectively. Each chapter includes a brief introduction followed by results and discussion, a concluding summary, and future considerations along with descriptions of any relevant preliminary studies investigated. Experimental and synthetic details for all films and compounds prepared are provided at the end of each respective chapter, and spectroscopic characterization (NMR and MS) is provided in Appendix A.
2: EXPERIMENTAL DETAILS
INTRODUCTION

This chapter presents experimental details regarding the surface characterization techniques used in the studies reported. Self-assembled monolayers and multilayered films were characterized by instrumental techniques to provide information about the film such as: wettability, chemical composition, coverage, and thickness. By analyzing the film at each stage of construction, and by comparing the differences from one layer to the next, the structure of the monolayer or multilayer can be determined. Techniques used in this thesis include: contact angle goniometry, infrared spectroscopy, x-ray photoelectron spectroscopy, cyclic voltammetry, and electrochemical impedance spectroscopy.

2.1. Contact Angle Goniometry.

Measurements of substrate wettability provide information about the type of functional groups exposed to the surface of the film. The sessile drop contact angle technique (used in this thesis) involves placing a drop of liquid, usually water, on the surface to be analyzed, and measuring the angle that the droplet makes with the surface. Non-polar terminal groups, such as alkyl groups of a SAM of dodecanethiol, will make a surface hydrophobic, and result in a high contact angle (112°). Polar functional groups, such as carboxylic acids of a SAM of mercaptoundecanoic acid, will make the surface hydrophilic, and result in a low contact angle (29°). The angle measured for a hydrophobic and hydrophilic surface is shown in Figure 2.1. An image of a contact angle goniometer used to make these measurements is presented in Figure 2.2. The instrument consists of a light source, a movable sample stage, a liquid dispensing system, and a digital camera. The instrument is interfaced to a personal computer for
Advancing and receding contact angle measurements can be used to determine the contact angle hysteresis, or the difference between advancing and receding contact angles. Advancing contact angles are measured as a drop of liquid is applied to surface immediately prior to separation of the drop from the dispenser and receding contact angles are measured as a drop of liquid is removed from a surface immediately prior to separation of the entire drop. Alternatively, advancing and receding contact angles can be measured using a tilting sample stage; the sample is tilted until the droplet moves across the surface and the advancing and receding angels are measured immediately prior to movement of the drop. The difference between the advancing and receding contact angle (hysteresis) provides information about the substrate such as roughness and homogeneity. A larger hysteresis is indicative of a rougher, inhomogeneous surface, and a smaller hysteresis is indicative of a smoother, uniform surface.

**Figure 2.1: Contact angle measurement.**
Sessile drop contact angle measurements were made using a Ramé-Hart Model 300 Goniometer (Netcong, NJ). Measurements were taken using 1 μL drops of deionized water deposited on the substrates using the Automated Dispensing System accessory coupled to the goniometer. Images were obtained by an integrated digital camera and the entire system was under computer control using Rame-Hart’s DROPlimage Standard (v. 2.0.10) software package. The software measures the contact angle once the liquid is dispensed using the contrast difference between the drop (dark) and the background (bright). Five measurements were taken per slide for five different samples and the results were averaged.

2.2. Grazing Incidence Infrared Spectroscopy.

Functional group analysis of monolayers on metal substrates was provided by grazing incidence infrared spectroscopy, also referred to in the literature as reflection-absorption infrared
spectroscopy (RAIRS) or polarized infrared external reflection spectroscopy (PIERS). In the experiment, the incident IR beam is reflected off the surface at a grazing angle (to enhance sensitivity) into the detector which measures the intensity of the reflected beam, as shown in Figure 2.3. The signal is inherently weak due to the small number of absorbing molecules, and sensitivity is also enhanced by purging the beam path with nitrogen during the experiment to limit interference of water or other vapors.

![Image of Grazing incidence IR measurement](image)

**Figure 2.3: Grazing incidence IR measurement.**

Not all absorption bands present in the monolayer are observed with this technique due to the orientation of the bonds relative to the surface. Only those vibrations with transition dipoles that have components perpendicular to the surface are observed. This effect, called the “metal-surface selection rule” determines which components of the molecule are IR active when observed by grazing incidence. A molecule absorbed on a metal surface induces a local, opposite charge in the substrate which enhances the transition dipoles oriented perpendicular to the substrate and cancels out the dipole for parallel orientations, as shown in Figure 2.4.
Figure 2.4: Molecular dipoles oriented perpendicular (left) and parallel (right) and the charges induced in the substrate; perpendicular dipoles are enhanced and IR active, parallel dipoles are negated and IR inactive.

Grazing incidence infrared spectra were obtained on the same instrument previously described equipped with a Thermo Nicolet grazing angle accessory in place of the ATR accessory. The incident IR beam was at 75 degrees to the gold substrates. Prior to measurement the optical path was purged with nitrogen for 30 minutes, and purging was continued during the experiments. A bare gold slide was used as the background, and a new background was collected immediately prior to each sample run. The scan range was from 4000 cm\(^{-1}\) to 600 cm\(^{-1}\), with 64 scans collected for each sample. The spectra were automatically corrected for H\(_2\)O and CO\(_2\), and a manual baseline correction was performed after each experiment.

2.3. X-ray Photoelectron Spectroscopy (XPS).

XPS measures the number and kinetic energy of electrons that are removed from a sample by X-ray irradiation in ultra-high vacuum (pressures lower than 1 \(\times\) 10\(^{-9}\) torr). The technique is
usually destructive to the sample. XPS provides the chemical composition of the material analyzed as electrons of each element have different energies, and the technique is used to determine the elemental components of self-assembled monolayers. A diagram of an XPS measurement of a SAM is shown in Figure 2.5, the instrument consists of an X-ray source, a collection lens (collects the emitted electrons), an electron energy analyzer (measures the kinetic energy of the emitted electrons), and an electron detector (counts the number of emitted electrons). 

Figure 2.5: XPS measurement of a SAM.

XPS analysis was performed by Anderson Materials Evaluation, Inc. (Columbia, MD) and the following experimental details were provided in their report. Samples were prepared, rinsed, and sealed in nitrogen purged vials during shipping for analysis. Spectra were collected on a Surface Science Instruments SX–100 X–ray photoelectron spectrometer equipped with a monochromatic Al Kα X–ray source (1487 eV), a wide-angle input lens, a hemispherical analyzer, and a
multichannel detector. An elliptical X–ray beam measuring 1.2 by 0.6 mm on the major and minor axes was used. Spectra were collected from 0 to 1100 eV at a step size of 0.5 eV using 32 scans of the energy range, a neutralizer electron beam energy of 2.4 eV, and a dwell time of 100 ms at each step. The analysis chamber was at less than $9 \times 10^{-10}$ torr during data collection. The C 1s primary component binding energy was set to 285.00 eV (found in unsaturated hydrocarbons and used as an internal standard) and all other binding energies were adjusted accordingly.

2.4. Cyclic Voltammetry (CV).

Cyclic voltammetry is used to examine the electrochemical properties of self-assembled monolayers, particularly their ability to restrict electron transfer. Cyclic voltammetry involves changing the potential across two electrodes in solution and monitoring the resulting current. If a redox active species is present in the electrolyte, the reversible oxidation and reduction will be observed as an increase in the anodic (oxidation) or cathodic (reduction) current. CV experiments are used to characterize the blocking or non-blocking nature of a SAM to this redox process; a well ordered monolayer forms an insulating layer and the redox process, and resulting current, is attenuated.

A gold slide, with a SAM or multilayered film attached, is used as the working electrode. Electron flow is between the working electrode and the counter electrode. The potential is measured between the working electrode and the reference electrode (which has a known, constant potential). The electrolyte contains the redox active species (such as ferricyanide) and supporting electrolyte that serves as a charge carrier. The cell is connected to a potentiostat.
which controls the potential between the working and reference electrode by passing current between the working and counter electrodes.

A CV profile of a SAM (dodecanethiol), along with that of bare gold for comparison, in the presence of ferricyanide is shown in Figure 2.6. The oxidation/reduction process \((\text{Fe}^{2+} \leftrightarrow \text{Fe}^{3+})\) is observed when an un-functionalized slide (bare gold) is used as the working electrode. When a monolayer is formed on the substrate, electron penetration to the surface is blocked, and the redox process is not observed (SAM). This insulating behavior is indicative of a well ordered film that has formed a complete (no defects) layer on the substrate.

![Figure 2.6: Cyclic voltammogram of a SAM on gold.](image-url)
Electrochemistry experiments were performed with a Gamry Instruments (Warminster, PA) Reference 600 Potentiostat/Galvanostat/ZRA. A three-electrode cell was used with the SAM functioning as the working electrode, a platinum wire counter electrode, and referenced against a saturated calomel electrode (SCE). An alligator clip connected the gold slide to the instrument, and a surface area of 1 cm\(^2\) was placed in the electrolyte solution. The electrolyte was an aqueous 1 mM potassium ferricyanide solution with 0.1 M potassium chloride as supporting electrolyte. Voltammograms were obtained from −0.3 V to 0.7 V at a scan rate of 50 mV/s.

2.5. Electrochemical Impedance Spectroscopy (EIS).

Impedance spectroscopy is used to examine properties of self-assembled monolayers including surface coverage, monolayer composition, and the ability of surface sensor molecules to selectively bind ions. Impedance spectroscopy involves applying a sinusoidal AC potential with a DC offset potential. The capacitive components of the system studied give sinusoidal current responses that are 90 degrees out of phase with the AC perturbation, and resistive components give current responses in phase with the perturbation. By fitting this response to an ideal system (or model circuit), the capacitive and resistive contributions can be de-convoluted, and their magnitudes can be estimated.

A film on gold can be described as a parallel plate capacitor which is composed of two conductive surfaces separated by an insulating layer. In this system the gold surface and the electrolyte solution are the conductive plates, and the monolayer is the insulator. The capacitance of a parallel plate capacitor is given by the equation:
\[ C = \frac{\varepsilon \varepsilon_0}{d} \times A \]

where \( C \) is the capacitance, \( \varepsilon \) is the dielectric constant of the film, \( \varepsilon_0 \) is the permittivity of free space (a constant), \( d \) is the separation of the plates, and \( A \) is the area. Changes in the thickness \( (d) \) of the film will result in changes in the capacitance which can be measured by impedance spectroscopy. Ion binding in SAMs can also be monitored by impedance measurements. Binding of metal ions to a monolayer increases the charge of the film, consequently increasing the dielectric constant \( (\varepsilon) \), and as a result, the capacitance.

Charge transfer resistance of the film can be examined if the impedance measurement is done in the presence of a redox species at the formal reduction potential of the probe. If the redox probe has access to the substrate, the charge transfer resistance will be low (ohms), and if the interfacial properties of the film are such that the probe cannot access the surface, the charge transfer resistance will be high (kohms to Mohms). Changes in the charge transfer resistance are indicative of changes in the structure of the monolayer. For example, a substrate that binds metal ions, will have an increase in charge transfer resistance upon ion binding (in the presence of a positively charged redox probe) due to electrostatic repulsion in the film.

Impedance measurements of monolayers on gold are usually modeled to Helmholtz (a solution resistance in series with the monolayer capacitance) or Randles (an additional charge transfer resistance element in parallel with the monolayer capacitance) equivalent circuits. A diagram showing Helmholtz and Randles circuit along with plots of the imaginary vs. real components of the overall impedance in these systems is shown in Figure 2.7. The choice of equivalent circuit
depends on experimental conditions such as supporting electrolyte and applied potential. Supporting electrolytes that contain redox active species necessitate the inclusion of a charge transfer resistance element in the equivalent circuit model, and monolayers on gold have been shown to behave like different electrical circuits depending on the DC potential used in the experiment.\textsuperscript{97}

A constant phase element (CPE) can be used to model SAM systems that deviate from ideal capacitive behavior. In Figure 2.7 curves A and C indicate ideal capacitive behavior for each circuit shown, while curves B and D show nonideal behavior that would be expected if capacitive defects are present in the layers. The inclusion of a CPE in the circuit model takes into account impedance behavior of the system that results in sinusoidal current responses that are between an ideal capacitor (90 degrees out of phase with the perturbation) and an ideal resistor (in phase with the perturbation). Capacitive values can be extracted from CPE fitting using a series of equations, however this form of capacitance has units that depend on the capacitive ideality and cannot be compared between different systems.\textsuperscript{109-111}

Impedance spectroscopy was performed with the same instrument used for cyclic voltammetry with the same electrode configuration (working, counter, and reference). A supporting electrolyte of 0.1 M tris(hydroxymethyl)aminomethane (TRIS) buffer (adjusted to pH 7.4 with concentrated HCl) that contained 1 mM hexaammineruthenium chloride (redox probe) was used for ion titrations. Impedance measurements were taken at the reduction potential of the redox probe \textit{vs.} the reference (\(-0.18\) V \textit{vs.} SCE, \(-0.1\) V \textit{vs.} plated silver, determined from CV measurements) with a 10 mV AC perturbation over a frequency range of 10 kHz to 100 mHz (10
points per decade). Aliquots of 0.1 M aqueous ion solutions (LiCl, NaCl, KCl) that contained 1 mM hexaamineruthenium chloride were added to change the ion concentration. Multiplexed experiments were performed with three working electrodes (in the same electrochemical cell) interfaced with a manual switch that allowed each electrode to be independently selected for measurement. Data was fit to a Randles equivalent circuit using complex non-linear least square fitting with Gamry Echem Analyst (v.5.50) software.

![Complex impedance plots for two types of equivalent circuit models showing ideal (A and C) and nonideal (B and D) capacitive behavior](image)

Figure 2.7: Complex impedance plots for two types of equivalent circuit models showing ideal (A and C) and nonideal (B and D) capacitive behavior ($R_{\text{soln}}$ = solution resistance, $R_{\text{ct}}$ = charge transfer resistance, $C_{\text{dl}}$ = monolayer capacitance, CPE = constant phase element).
3: **SURFACE MODIFICATION**
INTRODUCTION

Chemical surface modification is a method to alter substrate properties and introduce functionality into a system. The aim of this chapter is to briefly describe a ‘toolbox’ approach to surface modification that is used in the studies presented in this thesis. This approach employs simple molecular components (a molecular toolbox) that are either commercially available or easily prepared to fabricate surface based systems through self-assembly. Multilayered (and therefore multicomponent) films constructed using both covalent and non-covalent interactions are introduced as a method to incorporate new chemistry into an existing monolayer.

Covalent coupling reactions involve forming chemical bonds at reactive surface sites, similar to conventional organic synthesis, and recent reports have focused on chemical reactions on monolayer modified substrates.\textsuperscript{112-113} These descriptions of ‘reactions in two dimensions’ have demonstrated that monolayers with reactive sites can be further functionalized. Specifically, the functionalization of carboxylic acid surfaces by amide bond formation has been shown to be a way to add functionality to substrates that first involves activation of the acid using carbodiimide chemistry, followed by exposure to a compound with a free amine group. The resulting multilayered films retain properties of the monolayer such as stability and a high degree of order.

Incorporation of multiple molecular components into thin films can also be achieved by non-covalent assembly using metal-ligand interactions. Multilayered films fabricated by the sequential deposition of organic molecules and metal ions increase the complexity and functionality of the surfaces compared to SAMs alone.\textsuperscript{114-116} In addition to previous work in our
laboratory,\textsuperscript{117-118} other examples of such multilayered films have been reported. For example, Ulman \textit{et al}. fabricated multilayered films of mercaptoalkanoic acid and copper ions,\textsuperscript{119} and Brust \textit{et al}. used alkanedithiols acid and copper ions.\textsuperscript{120} Other groups have demonstrated assembly of multilayered films based on the interaction of copper, zirconium and other ions with ligands containing sulfur, carboxylic acids, and phosphates.\textsuperscript{121-122} An advantage of this method of assembly is that ordered films with complex structures can be constructed from simple molecular building blocks in a controlled manner with minimum synthetic effort.

This chapter is divided into three sections. The first is a brief discussion of functional SAMs used in this thesis that expands on the description of how our laboratory approaches surface modification introduced in Chapter 1. Multicomponent films formed covalently and non-covalently are discussed in the two following sections, respectively. The purpose of this chapter is to provide a comprehensive description of the different types of surface modification methods used in the work presented.
RESULTS AND DISCUSSION

3.1. Monolayers.

The first step in the surface modification process introduced in Chapter 1 is to select attachment chemistry for the substrate; the studies reported in this thesis are performed on gold, and thiols groups were used for the reasons described previously. A selection of functional thiol compounds with different terminal groups (CH$_3$, COOH, OH, NH$_2$, etc.) and different alkyl chain lengths (3 to 18) is available commercially and serve as a foundation for a molecular ‘toolbox’. As described below, monolayers with reactive or complexation sites are used as a scaffold for multilayered film assembly.

Synthetic routes to functional thiol compounds for monolayer preparation are also presented in this thesis. In the interest of using simple molecular components that are prepared quickly, all synthetic routes are limited to 4 or less steps; one step preparations are preferred and used in the majority of the studies presented. A molecular component for surface modification was prepared that included a photoactive group and the one step procedure is described in Chapter 4, Section 4.1. This compound was also demonstrated to be a scaffold for fabricating multilayered films both covalently and non-covalently (see below). Molecular components for surface based ion recognition were also prepared using a one step synthetic process (Chapter 6, Section 6.7) New functional molecular components for thin films that are either photoactive or ion binding are also proposed in the future work section of Chapter 6.
3.2. Covalently Coupled Multilayered Films.

Multilayered films formed by covalent coupling were fabricated in patterns and are the subject of Chapter 4. Covalently assembled films were also used to generate substrates with regions that promoted nerve cell adhesion, see future work in Chapter 4. Amidation reactions on surfaces proceed with high yield and can be performed using either free amine or carboxylic acid surface sites. Covalent coupling by amide bond formation is a three step process.

SAMs with carboxylic acid terminal groups are activated with EDC/NHS toward amide bond formation. Following activation, exposure to a solution of a compound with a free amino group results in a covalent surface coupling reaction. The mechanism for amide bond formation using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) activation on a surface is shown in Figure 3.1. EDC first reacts with the free carboxylate to form an unstable amide-reactive intermediate, which then undergoes nucleophilic substitution with NHS to form a semi-stable amine-reactive NHS ester. The NHS ester is exposed to a solution of a free amine compound (R-NH₂) which displaces the NHS moiety by nucleophilic substitution, forming an amide bond.

EDC/NHS activation of a carboxylic acid terminated monolayer is confirmed by contact angle and grazing IR measurements; a comprehensive description is provided in Chapter 4, Section 4.8. Deposition of fluorophores by amide bond formation on activated monolayers is detailed in Section 4.9, along with characterization data. The process can also be performed in reverse; i.e. activation of a carboxylic acid group in solution and exposure to a monolayer with a free amine group to form an amide bond and multilayered film. A method to couple a photoactive
compound to a surface using this approach is described in the future work section of Chapter 4.

![Mechanism for surface amide bond formation](image)

**Figure 3.1: Mechanism for surface amide bond formation.**

3.3. *Non-covalently Coupled Multilayer Films.*

Multilayered films were assembled using non-covalent interactions with SAMs possessing a metal ion binding site; terminal dicarboxypyridine and carboxylic acid groups are used in the work presented. A diagram of these two terminal groups complexed to metal ions on a substrate is shown in Figure 3.2. Following monolayer formation, the SAM is exposed to a transition metal ion solution to form a complex with the binding site of the film. Another organic ligand with a metal ion complexation site is used to ‘cap’ the metal ion and complete the multilayered film. This method can be repeated in a layer-by-layer process to generate films with multiple,
alternating organic and metal components.

Metal ion complexation is confirmed by contact angle, and CV measurements. Contact angles of dicarboxypyridine SAMs decrease from ~ 75° to values between 55° and 60°; contact angles of carboxylic acid SAMs increase from ~ 15° to comparable values to above. Electrochemical measurements of metal capped films show non-blocking behavior to a redox process in solution (identical to bare gold) which is discussed in detail, Chapter 5, Section 5.4.

Figure 3.2: Non-covalent metal-ligand interactions of a dicarboxypyridine SAM (left) and a carboxylic acid SAM (right).

It is noted that in non-covalent, multilayered systems the films are charge balanced by the presence of counter ions on the surface. The diagram in Figure 3.2 is not intended to show the
nature of the metal-ligand interaction, but rather to simply indicate that the multilayered assembly proceeds when a metal ion interacts with a surface based binding site. The counter ions (from the anion of the metal salt used) will bind to the film to balance the charge and result in a neutral film. For example, when using Cu(II) ions it is likely that the carboxylic acid substrate is deprotonated when the metal ion binds and that a negative counter ion is present at each binding site to charge balance the film.

Non-covalent interactions were used to fabricate films that underwent wettability changes when exposed to light and is the subject of Chapter 5. Non-covalent assembly was also attempted for patterned carboxylic acid substrates (described in Chapter 4, Section 4.7) and future considerations for these systems are detailed at the end of the chapter.
**Conclusions**

A ‘toolbox’ approach to surface modification allows complex systems to be fabricated from simple components. Molecular constructs used for self-assembly in this thesis are prepared with limited synthetic effort and can be used for more than one study. Multicomponent films are assembled using both covalent and non-covalent approaches. Covalent coupling is through amide bond formation and non-covalent coupling utilizes metal-ligand complexes. Using a set of molecular building blocks and a stepwise approach to surface functionalization, patterned systems, systems with switchable wettability, and systems for molecular recognition have been developed. The following three chapters discuss these applications in detail.
4: **Surface Patterning**
INTRODUCTION

This chapter describes a surface patterning method with the goal of generating a photoactive surface that can be further functionalized in specific regions to form a stable, multilayered film. The method is designed to be repeatable on the same substrate; a multi-step process is used to irradiate and chemically modify one area of a substrate, and then the irradiation and modification procedure is repeated in another area of the substrate. Surface patterning, and specifically the ability to sequentially pattern the same substrate, is of interest for generating both multi-analyte sensing surfaces and surfaces that control neuronal cell adhesion and growth.\textsuperscript{123}

In order to generate a multi-analyte sensing platform, a method to attach multiple sensing moieties to the same surface based device is needed. Multi-analyte sensing surfaces are of interest for developing portable blood sensors that detect electrolytes, glucose levels, and protein levels in the same device. A multilayered patterning method that allows attachment of a selective sensor to one portion of a deprotected surface, and then repetition of the process in other areas will produce substrates with regions that respond to different analytes.

Chemically modified surfaces that control protein adhesion as well as cell attachment and growth are used for biomedical applications.\textsuperscript{50,124-125} Patterned monolayers that promote cell adhesion in specific regions of a substrate and those that inhibit adhesion in others can be used to create surface based cellular networks.\textsuperscript{126} Specific interest is in the patterned growth of nerve cells for reconnecting neuronal networks damaged by injury. By creating patterned surfaces that promote cell adhesion, an artificial scaffold that directs cell growth can be developed with the potential to
be implanted in an area of nerve cell damage to re-establish connectivity for sensation and function.

SAM patterning techniques to generate multi-component films have been developed to create chemically modified surfaces that exhibit different properties in defined areas. Approaches to generating surface patterns include depositing a monolayer in specific regions using microcontact printing\textsuperscript{54} and dip-pen nanolithography,\textsuperscript{127} or removal of the monolayer from specific regions using high energy sources such as lasers\textsuperscript{128} and electron beams.\textsuperscript{129} Other methods (discussed below) have used photo-reactive compounds deposited as SAMs to generate surface patterns. SAMs of alkanethiols on gold have been shown to be stable under irradiation by ultraviolet light at wavelengths as short as 254 nm.\textsuperscript{45-46}

Zhao \textit{et al.} have demonstrated a method for directing flow patterns inside silica microchannels using a self-assembled monolayer fabricated from a compound with a photolabile protecting group.\textsuperscript{61,130-132} Nitroveratryloxycarbonyl (NVOC) protecting groups have also been used to generate quinone, oxyamine, and amine reactive surface sites that can then be further functionalized.\textsuperscript{133-136} Although these approaches have all successfully patterned surfaces, drawbacks include: non-reactivity of the patterned substrates toward further functionalization, deprotection steps necessitating solvent to facilitate the photoreaction or to limit competing reactions, and the inability to further pattern the surfaces.

Previous work in our laboratory has focused on using chemical surface modification for applications such as controllable switchable wettability, photovoltaics, and selective ion
sensing. However, none of this previous work has involved employing surface patterning methods. The utility of these types of systems can be expanded by selectively patterning substrates and attaching specific moieties to defined regions of the same surface. This chapter describes how photo-patterning has been combined with covalent surface coupling to create multi-component films. Multilayered film assembly with this method allows a surface to be patterned multiple times. Advantages of this method over other types of SAM patterning include: the ability to achieve complete deprotection of the photolabile group in air in the absence of solvent, the surface activation step makes the patterned areas reactive toward any compound with a free amine group, and the multilayered films created are stable to further patterning.

Photolabile head groups can also be used to generate sufficient surface separation for multilayer film assembly. Following removal of a protecting group, the reactive site spacing is increased compared to that of a conventionally prepared monolayer, which allows for attachment of subsequent functionalities that undergo a conformational change and alter the properties of the surface, such as wettability. This method will be discussed in detail in the next chapter.

Overall, this system is a universal method for selectively attaching moieties with free amine groups to site specific areas of a surface. To demonstrate this patterning method, multiple fluorophores were attached to the substrate and the resulting patterns imaged with fluorescence microscopy. Each modified substrate has also been fully characterized to confirm the step-by-step multilayered film assembly process.
RESULTS AND DISCUSSION


An alkanethiol, 2-nitrobenzyl-11-mercaptoundecanoate (photolabile compound), with an o-nitrobenzyl protecting group was synthesized and used as the base for multilayered film assembly. The synthetic procedure used to generate the photolabile compound is shown in Scheme 4.1, and synthetic details are described at the end of this chapter. The carboxylic acid adds to one of the double bonds of the dicyclohexylcarbodiimide (DCC) to form and o-acylisourea, which is a good leaving group. The alcohol then adds to the carbonyl group of the o-acylisourea, forming a tetrahedral intermediate which rapidly dissociates into an ester and dicyclohexylurea. Following work-up and purification, the final structure was confirmed by $^1$H and $^{13}$C NMR analysis as well as mass spectroscopy (all spectra are provided in Appendix A).

Scheme 4.1: Synthesis of 2-nitrobenzyl-11-mercaptoundecanoate (photolabile compound).
4.2. SAM Deposition and Characterization.

Monolayers of the photolabile compound were deposited on piranha cleaned gold substrates from ethanolic solution. Successful SAM formation was confirmed by contact angle, grazing incidence IR, cyclic voltammetry, and XPS measurements. Although arrangement of the monolayer is not precisely known, it is expected that a portion of the benzyl ring will be exposed to the surface, making it hydrophobic. SAMs of the photolabile compound produced a sessile water droplet contact angle of $72^\circ \pm 2.0^\circ$, consistent with a hydrophobic terminal nitrobenzyl moiety. A grazing incidence infrared spectrum of the protected monolayer is shown in Figure 4.1. The major IR absorption frequencies of the SAM are also observed in a solid sample of the photolabile compound examined by ATR (Figure 4.2). The CH\textsubscript{2} stretching signals at 2920 cm\textsuperscript{-1} and 2851 cm\textsuperscript{-1} are indicative of a crystalline arrangement of alkyl chains.

Cyclic voltammetry was used to examine the coverage and organization of the monolayer. A CV scan of the protected monolayer (in the presence of ferricyanide as the redox probe) is shown in Figure 4.3, along with that of a bare gold surface for comparison. The attenuated current in the CV, as compared to that of bare gold, is indicative of a well ordered, insulating monolayer.

XPS analysis was performed on samples following monolayer deposition and the data confirms successful SAM formation. The XPS spectrum is shown in Figure 4.4 and a summary of the elements detected, binding energy, and atomic percentages for each element in the SAM is provided in Table 4.1. The elements observed are expected for a SAM of the photolabile
compound on gold with the exception of trace amounts of Sn and I, which arise from the substrate preparation process (float glass is prepared by floating the glass on a layer of molten tin which also has iodine present as confirmed by the supplier, Evaporated Metal Films) and are also seen in an XPS spectrum of the bare substrate (shown in Figure 4.5 and summarized in Table 4.2). The presence of the C, O, and S on the surface is not surprising as gold films commonly have a very high surface concentration of organic materials. Remaining adsorbed ethanol from the substrate rinsing step contributes to the C and O signals observed. Atmospheric carbon compounds (called adventitious carbon) will also adsorb on any surface exposed to the air, and sulfur compounds have a very high affinity for gold substrates even at low concentrations.

From the XPS data, a molecular formula for the monolayer of C\textsubscript{18}O\textsubscript{4.8}N\textsubscript{1.2}S\textsubscript{0.6} (not accounting for hydrogen) is obtained and is consistent with the expected chemical formula of C\textsubscript{18}O\textsubscript{4}NS. The differences in the measured and expected molecular formulas are due to the depth of the individual elements in the film; electrons from deeper in the film are more likely to have inelastic collisions and lose energy prior to reaching the detector, consequently attenuating their signals. XPS analysis indicates that the film is oxygen and nitrogen rich and sulfur deficient, which is consistent with the SAM being bonded to the gold through the sulfur and the rest of the molecule oriented away from the surface, terminating with the o-nitrobenzyl group. The binding energy for the nitrogen 1s signal at 406.37 eV indicates that the nitrogen is present as an organic nitro (C-NO\textsubscript{2}) group.\textsuperscript{83}
Figure 4.1: Grazing incidence IR of the photolabile compound deposited as a SAM.

Figure 4.2: ATR IR spectrum of solid 2-nitrobenzyl-11-mercaptoundecanoate (photolabile compound).
Figure 4.3: Cyclic voltammogram of a SAM of the photolabile compound, bare gold is shown for comparison.

Figure 4.4: XPS Spectrum of SAM of the photolabile compound.
Table 4.1: Summary of XPS data for a SAM of the photolabile compound.

<table>
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<tr>
<th>XPS Line</th>
<th>BE (eV)</th>
<th>Atom %</th>
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<tr>
<td>C 1s</td>
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<td>Au 4f</td>
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<td>S 2p</td>
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<td>O 1s</td>
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</tr>
<tr>
<td>I 3d5</td>
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<td>0.15</td>
</tr>
<tr>
<td>N 1s</td>
<td>406.37</td>
<td>4.03</td>
</tr>
</tbody>
</table>

Figure 4.5: XPS Spectrum of a bare gold substrate.
Table 4.2: Summary of XPS data for a bare gold surface.

<table>
<thead>
<tr>
<th>XPS Line</th>
<th>BE (eV)</th>
<th>Atom %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1s</td>
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</tr>
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</tr>
<tr>
<td>I 3d5</td>
<td>619.21</td>
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</tr>
</tbody>
</table>

4.3. Photodeprotection.

α-Nitrobenzyl moieties are known as photolabile protecting groups that undergo Norrish-type II reactions when excited by ultraviolet light.\textsuperscript{143-144} Norrish reactions, first reported by Ronald George Wreyford Norrish in 1937, are photoreactions that occur in carbonyl compounds and are divided into two types (I and II).\textsuperscript{145} Norrish-type I reactions involve cleavage of aldehydes and ketones into free radicals. A Norrish-type II photo-cleavage (occurring in this case) involves the excited nitro group abstracting a proton from the methylene carbon on the aromatic ring forming a radical. The radical is resonance stabilized by a five-membered ring intermediate which rapidly decomposes to an aldehyde and a carboxylic acid. A mechanism for the photocleavage is shown in Scheme 4.2.
Scheme 4.2: Norrish-type II photo-cleavage.
Absorption spectra were obtained in ethanolic solution for the photolabile compound (2-nitrobenzyl-11-mercaptoundecanoate) prior to irradiation. The compound has a maximum absorbance peak at 257 nm with a shoulder extending out past 350 nm, Figure 4.6. A 300 nm lamp was chosen for irradiation experiments to remove the protecting group, but not destroy the monolayer (thiol gold bonds can be oxidized by deep UV light). A Rayonet reactor equipped with a 30 watt 3000 Å (300 nm) mercury lamp was used for all photo-deprotection experiments. An emission profile of the lamp is provided as Figure 4.7. The profile of the lamp has a broad emission profile centered slightly above 300 nm, and a number of sharp mercury lines are seen at higher wavelengths. It is noted that the photomasks used for surface patterning experiments cut off light below 300 nm and the lamp profile demonstrates sufficient emission above 300 nm to remove the photolabile group. Following irradiation, the absorbance peak at 257 nm is no longer observed (in solution), indicating a photoreaction has occurred.

![Absorbance spectra of the photolabile compound in solution (EtOH).](image)

**Figure 4.6:** Absorbance spectra of the photolabile compound in solution (EtOH).
4.4. SAM Photodeprotection.

The structure and photo-product of the photolabile compound on a surface is shown in Figure 4.8 (this is an idealized representation for clarity and not a depiction of the molecular arrangement on the surface). Following deprotection, a carboxylic acid terminated layer will be exposed to the surface, providing a reactive site for further functionalization. The surface can also be deprotected in a pattern, leaving defined areas available for covalent coupling.
Deprotection of the SAM was confirmed by contact angle, grazing incidence IR, cyclic voltammetry and XPS. After irradiation and removal of the protecting group, the contact angle of the surface decreases from $72^\circ \pm 2.0^\circ$ to $16^\circ \pm 3.0^\circ$, consistent with an acid terminus on the surface. Contact angles of the deprotected substrate are also pH sensitive; values of ~ $40^\circ$ were obtained using acidic solution (pH 2) and values of ~ $10^\circ$ were obtained using basic solution (pH12). It is noted that these values are constituent with those obtained from a film of mercaptoundecanoic acid (COOH terminated) when examined with different pH soilutions, and it is also noted that the monolayer (unirradiated) shows no pH dependence of contact angle.
A grazing incidence infrared spectrum of the deprotected monolayer is shown in Figure 4.9 along with the protected monolayer for comparison. Removal of the protecting group following irradiation is confirmed by comparing the spectra with that of the SAM. The signal at 1535 cm$^{-1}$ is no longer observed (the nitro group in the monolayer), indicating that the $\alpha$-nitrobenzyl group has been cleaved. The carbonyl stretching frequency shifts from 1744 cm$^{-1}$ to 1715 cm$^{-1}$ after irradiation, which is consistent with the original ester being converted to a carboxylic acid. Stretching frequencies for the alkyl chain are unchanged.

![Grazing incidence IR of a SAM (top) and irradiated SAM (bottom) of the photolabile compound.](image)

**Figure 4.9:** Grazing incidence IR of a SAM (top) and irradiated SAM (bottom) of the photolabile compound.
Cyclic voltammetry was used to confirm the presence of a well ordered monolayer following exposure to ultraviolet light and removal of the protecting group. The CV profile for an irradiated monolayer is shown in Figure 4.10 along with that of bare gold for comparison. Following irradiation, the surface remains blocking (no current is seen for the redox of ferricyanide), indicating that the exposure to ultraviolet light has not removed the monolayer.

![Cyclic voltammogram of the deprotected monolayer, bare gold is shown for comparison.](image)

**Figure 4.10**: Cyclic voltammogram of the deprotected monolayer, bare gold is shown for comparison.

After exposing the monolayer to UV light, samples were re-examined by XPS to confirm removal of the protecting group. The XPS spectrum of an irradiated SAM is shown in Figure 4.11 and a summary of the elements detected, binding energy, and atomic percentages for
each element is provided in Table 4.3. It is evident from the XPS data that the irradiation removed the \textit{o}-nitrobenzyl protecting group from the monolayer. A complete absence of nitrogen is observed in the XPS spectrum of the irradiated SAM, and also the atomic percentages of carbon and oxygen have decreased compared to the monolayer as expected due to the cleavage of the protecting group as a nitrobenzyl aldehyde. Once again, the trace amounts of Sn and I arise from the underlying substrate.

![Figure 4.11: XPS of an irradiated SAM of the photolabile compound.](image)

Figure 4.11: XPS of an irradiated SAM of the photolabile compound.
Table 4.3: Summary of XPS data for a SAM of the photolabile compound following irradiation.

<table>
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<th>Atom %</th>
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<td>S 2p</td>
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<td>Sn 3d</td>
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<td>O 1s</td>
<td>532.87</td>
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<tr>
<td>I 3d5</td>
<td>619.10</td>
<td>0.17</td>
</tr>
</tbody>
</table>

4.5. Diffraction.

Initial patterning experiments resulted in significant distortion due to diffraction. Photolithography can be divided into three types: contact, proximity, and projection.\textsuperscript{146-147} In contact lithography the photomask is in firm contact with the substrate that is being patterned. Flexible photomasks are used along with mask aligners and vacuum systems to tightly adhere the photomask to the substrate, resulting in a 1:1 pattern transfer that is not limited by diffraction. Proximity lithography involves having the mask close to the substrate (separated by 2 to 20 microns). The pattern is once again transferred in a 1:1 ratio, however near-field diffraction limits resolution. This type of diffraction occurs when light passes through a small aperture and bends, resulting in a diffraction pattern that differs in size and shape from the aperture in the near-field (in the range of the separation distance in proximity lithography).\textsuperscript{148-149} Projection lithography involves the use of optics to project a pattern onto the substrate; the mask is not in contact with the surface and the transfer ratio is controlled by the optical setup.
For this work, a glass photomask with a chrome coating that provides the pattern was used. Placing the photomask on top of the SAM modified substrate for photo-patterning results in proximity lithography, as the separation has micron dimensions. The mask and substrate are metal coated glass, both hard surfaces, and a separation of micron dimensions is expected given any surface imperfections or small particulate contaminants trapped within the interface. Near-field diffraction becomes a problem when trying to obtain precise patterned dimensions. A diagram of the effect of diffraction on the resolution of a patterned surface is shown in Figure 4.12.\textsuperscript{146}

![Figure 4.12: Effect of near-field diffraction.\textsuperscript{146}](image-url)
Diffraction distorts the surface patterns which results in a higher degree of intensity at the edges of the pattern, as well as light exposure outside the dimensions of the mask, as seen in the plot in Figure 4.12. The greater the separation between the mask and surface, the more diminished the quality of the pattern. Experiments involved irradiating the surface in solution with the photomask placed on top. This method resulted in a high degree of diffraction due to the separation between the mask and substrate and the patterns generated did not maintain the features of the photomask. For example, a 100 µm square pattern was distorted; the overall dimensions of the pattern increased resulting in features approximately 10 µm larger than the mask when observed by microscopy.

To limit near-field diffraction, a fixture was designed to clamp the mask to the substrate in order to decrease the separation distance. It was also determined that the deprotection could be performed in the absence of solvent (from contact angle and IR data), meaning the interface between the mask and substrate could be minimized. Although it is still technically proximity lithography, the minimization of the space between the mask and substrate resulted in pattern transfer with a limited amount of distortion due to diffraction.


SAMs of the photolabile compound were irradiated through a photomask to generate patterned surfaces. A SAM modified substrate was patterned by clamping a photomask (100 µm x 100 µm squares) to the surface, and irradiating at 300 nm through the mask. After irradiation the resulting surface pattern can be observed as the protected and deprotected regions have a significant difference in wettability. By coating the surface with ethanol and allowing a portion
to evaporate, a droplet pattern is observed on the substrate; an optical micrograph is shown in Figure 4.13.

![Image of ethanol droplets on a modified surface](image)

**Figure 4.13:** Optical micrograph of ethanol droplets on a surface modified with a SAM of the photolabile compound after irradiation at 300 nm through a 100 µm square photomask (shown in inset), scale bar represents 200 µm.

The wettability difference between irradiated and unirradiated areas of the same surface was also demonstrated by placing aqueous dye solutions on the surfaces and observing that the aqueous solution is confined to the hydrophilic regions. Simple photomasks were prepared using black electrical tape (2 mm wide strips and 2 mm wide cuts-outs of the letters WPI) placed on glass slides. SAMs of the photolabile compound were prepared on gold substrates and irradiated through these photomasks. Application of polar solutions (aqueous solutions of fluorescein, coumarin, and rose bengal) and non-polar solvent (hexadecane) to the patterned surface using a
pipette demonstrates the wettability difference as shown in the images in Figure 4.14. The aqueous fluorescent dyes remain in the irradiated (hydrophilic) regions and the hexadecane remains in the unirradiated (hydrophobic) regions.

**Figure 4.14:** Images of surfaces patterned through masks with cut-outs of the letters WPI (left, 2 mm dimensions), and 2 mm stripes (right), following application of aqueous dye to the irradiated regions and hexadecane to the unirradiated surrounding areas. The dye solutions (fluorescein - green, coumarin - blue, and rose bengal - red/orange) demonstrate the areas of the substrate that have been irradiated.

4.7. *Non-Covalent Assembly.*

The photolabile compound used in this study leaves a carboxylic acid terminus on the substrate after removal of the protecting group. Previous work has demonstrated the ability to fabricate multilayered systems based on metal-ligand interactions in thin films and metal ions have been shown to form complexes with carboxylic acid terminated monolayers. Patterned multilayered film assembly was attempted using non-covalent interactions. A series of metal ions were tested with this system including: Ag(I), Cu(II), Co(II), Ni(II), Zn(II), Hg(II), Pb(II), Cr(III), Fe(III), Ru(III), Eu(III), and Pb(IV). Metal ions were selected to test differences in oxidation state and ionic radius on film assembly. For multilayered films to be constructed non-
covalently, the metal ion must form a complex with the deprotected areas, but not interact with the remaining protected areas of the surface.

Films of the unirradiated SAM were exposed to metal ions to determine if any changes occurred based on the characterization data. Although it was not possible to determine the nature of the interaction, it was discovered that exposure to metal ions changes properties of the protected film. For example, treating the unirradiated SAM with Ag(I), Cu(II), Zn(II), Hg(II), Fe(III), or Ru(III) resulted in films that were no longer blocking when examined by CV. Certain metal ions [Cu(II), Fe(III), and Ru(III)] destroyed patterned SAMs as evidenced by wettability tests (a droplet pattern was not observed on the substrate after metal ion exposure). Exposure to metal ions does not remove the photolabile group (the nitro peak was observed by IR after exposure), so the ions are either specifically or non-specifically interacting with the protected film, which is a disadvantage for multilayered assembly.

Lead (Pb) ions seemed promising in that patterns were retained after exposure, and the electrochemical properties of the film were unchanged (films remained blocking). XPS was performed on an unirradiated SAM that had been exposed to lead, which showed that lead is present on the substrate. It was not possible to determine how lead ions were bonded to the film and if the interaction was specific (complexation) or non-specific (deposition or penetration). Because the exposure to metal ions affects the unirradiated SAM, covalent assembly was investigated to generate patterned multilayered films.
4.8. *Surface Activation.*

As described in Chapter 3, Section 3.2, SAMs with carboxylic acid terminal groups are activated with EDC/NHS toward amide bond formation.\textsuperscript{112-113} Surface activation was confirmed by characterization on slides that had been irradiated completely (no photomask used) and treated with EDC/NHS. EDC/NHS activated surfaces demonstrated an increase in hydrophobicity, resulting in a contact angle of $44.8^\circ \pm 2.0^\circ$. This value is comparable to measurements taken on other carboxylic acid terminated films activated with EDC/NHS, and the decrease in wettability is indicative of the carboxylate terminated surface being replaced with a NHS ester functionality.

Grazing incidence IR shows the presence of NHS on the activated surface and also confirms that treatment with EDC/NHS does not affect the unirradiated surface. IR spectra of surfaces exposed to EDC/NHS are shown in Figure 4.15 (SAM) and Figure 4.16 (irradiated SAM). Analysis of the protected SAM exposed to EDC/NHS shows that the carbonyl of the ester (1743 cm$^{-1}$) and the nitro group (1535 cm$^{-1}$) are unchanged compared to the monolayer (see Figure 4.1), confirming that the protected monolayer is present on the surface. The only difference in the IR spectra between the SAM and the SAM exposed to EDC/NHS is an increase in the CH$_2$ stretching frequencies of the alkyl chain. The higher wavenumbers for these signals are indicative of a liquid-like arrangement of the alkyl chains on the surface. This observation is due to the SAM being exposed to water during EDC/NHS activation; water has penetrated the film and caused a reorientation of the alkyl chains, however a SAM of the photolabile compound is still present.
IR spectra of the deprotected SAM exposed to EDC/NHS demonstrate that the surface has been activated (Figure 4.16). The signal at 1715 cm\(^{-1}\) present for the deprotected monolayer is not observed, indicating that a carboxylic acid is no longer present, as expected if the carboxylate has formed an ester bond. The three peaks and their relative intensities at 1816 cm\(^{-1}\), 1789 cm\(^{-1}\), and 1744 cm\(^{-1}\) are indicative of NHS attachment, consistent with previously published results.\(^{75,150}\) Exact assignment of these signals to the different carbonyl stretching modes of the NHS functionality is controversial as both the highest and lowest frequency signals have each been assigned to the ester carbonyl stretch in different reports.\(^{75,78-79}\) The stronger absorption at 1744 cm\(^{-1}\) is most likely due to the carbonyl stretching of the NHS ester, as an ester signal at 1744 cm\(^{-1}\) was observed for the monolayer. The signal at 1646 cm\(^{-1}\) has been attributed to the
carbonyl stretch of trace amounts of urea on the surface (the product of NHS displacing EDC in the activation step), but can also be attributed to absorbed water as the substrates were thoroughly rinsed after activation. As in the unirradiated monolayer, the CH$_2$ stretching signals of the alkyl chain have increased in frequency due to reorganization during the activation step. Grazing incidence IR analysis demonstrates successful surface activation of the deprotected monolayer, and also that the protected areas of the surface are not affected.

Figure 4.16: Grazing incidence IR of the irradiated SAM activated with EDC/NHS.

Cyclic voltammetry of NHS activated surfaces confirms that a SAM remains on the substrate after EDC/NHS exposure; the substrate remains blocking to a redox process in solution (Figure 4.17). The increase in current in comparison to the monolayer seen after the activation step indicates that slow electron penetration through the layer is occurring (see more detailed
This observation is consistent with the grazing IR data that showed a less crystalline (liquid-like) arrangement of the monolayer after activation.

Figure 4.17: CV analysis of a SAM activated with EDC/NHS.


To demonstrate multilayered surface patterning, two commercially available compounds were used to attach to the patterned surfaces, rhodamine 110 (6-amino-9-(2-carboxyphenyl)-3H-xanthen-3-iminium chloride) and cresyl violet 670 (9-amino-5H-benzo[a]phenoxazin-5-iminium perchlorate). Chemical structures of rhodamine 110 and cresyl violet 670 are shown in Figure 4.18; both fluorophores offer two possible sites for amide bond formation due to
resonance through the aromatic system (the charged group can be at either end of the molecule). In ethanolic solution (used for deposition of the fluorophores) the charged nitrogen shown in the chemical structure is likely deprotonated resulting in a neutral compound with one free primary amine group. These compounds are fluorescent in different areas of the spectrum (rhodamine 110 in the green, and cresyl violet 670 in the red), and can be used to provide visual evidence of patterned multilayered film assembly. Absorption and fluorescence emission spectra for the two fluorophores in solution (EtOH) are provided in Figure 4.19. The maximum emission intensity differs by more than 100 nm and the majority of the emission for each fluorophore lies within the appropriate band pass filter. It is also noted that the absorbance of each fluorophore matches with the profile of each of the respective excitation filter.

Figure 4.18: Chemical structures of rhodamine 110 (left) and cresyl violet 670 (right).
Amide bond formation and fluorescent ligand attachment was confirmed on substrates that had been irradiated completely (no photomask), activated with EDC/NHS, and exposed to solutions of the fluorophores to form a multilayered film. Contact angles for rhodamine 110 functionalized surfaces were measured to be $48.4^\circ \pm 2.5^\circ$, and contact angles for cresyl violet 670 were found to be $49.2^\circ \pm 3.0^\circ$. Analogous values for the films are expected since each fluorophore has a similar structure, and the angles are comparable to those measured for monolayers with an amine terminated functionality ($46^\circ \pm 2.0^\circ$). Formation of an amide bond at one end of each fluorophore results in the free amine group at the other end being exposed to the surface.
Grazing incidence IR also confirms the presence of the fluorophores on the substrate. IR spectra for the activated monolayer exposed to rhodamine 110 and cresyl violet 670 are shown in Figures 4.20 and 4.21, respectively. IR spectra for both fluorophore functionalized films show a broad N–H stretch between 3200 cm$^{-1}$ and 3500 cm$^{-1}$, centered at 3370 cm$^{-1}$ and 3329 cm$^{-1}$ for rhodamine 110 and cresyl violet 670 films, respectively. These signals are in the same region as the N–H stretching peaks observed in IR analysis of the bulk compounds, shown in Figures 4.22 and 4.23. For each film the N–H absorption is broad due to the multiple stretching modes present; each surface has a free amine as well as an amide bond contributing to the absorption at these frequencies.

A new signal, not present in the IR spectra of either the activated substrate or the solid samples, is observed at 1688 cm$^{-1}$ for surfaces exposed to rhodamine 110 and at 1691 cm$^{-1}$ for surfaces exposed to cresyl violet 670. These absorptions are assigned to the C=O stretch of the amide bond and are consistent with the IR absorption for carbonyls of an analide functionality (between 1680 cm$^{-1}$ and 1700 cm$^{-1}$), now present on each fluorophore modified surface. Each spectra also shows absorptions at approximately 1640 cm$^{-1}$ and 1595 cm$^{-1}$ (observed at the same locations in the solid compounds), and are due to either C–N or aromatic C=C vibrations. Both fluorophore modified films retain a signal at 1745 cm$^{-1}$, indicating that an amide bond has not formed at each reactive surface site. This observation is not surprising as both fluorophores are composed of multiple rings with considerable bulk compared to the activated surface sites, and steric hindrance limits the nucleophillic substitution reaction from occurring at each location. The carbonyl stretch at 1745 cm$^{-1}$ is attributed to either remaining NHS ester moieties or
hydrogen bonded carboxyl groups (if the ester has hydrolyzed), and retention of this signal has been observed in reports of amide bond formation using this method.\textsuperscript{75} It is noted that surfaces functionalized with rhodamine 110 have a broad absorption in the entire region between 1670 cm\textsuperscript{-1} and 1750 cm\textsuperscript{-1}. Rhodamine 110 also has a carboxylic acid functionality that will absorb in this area which is seen at 1703 cm\textsuperscript{-1} in the spectrum of the solid sample.

Cyclic voltammograms of each functionalized surface are shown in Figures 4.24 and 4.25 along with a profile of a bare gold substrate for comparison. CVs confirm the presence of a monolayer, as evidenced by the reduced current compared to the profile of bare gold. The increase in current, in comparison to the monolayer, is because the multilayered film is not as ordered as the monolayer; slow electron penetration through the film is occurring and this behavior is observed in other multilayered systems.\textsuperscript{117-118,137} Bulky terminal groups bound to the film likely decrease the order of the system as the film reorganizes once the multilayered film forms; in effect, the multilayered system is not a well organized arrangement of simple alkyl chains, but a more disorganized, loosely packed arrangement. These electrochemical observations are in agreement with the grazing IR data that indicates a liquid-like arrangement of the backbone chain upon multilayered assembly.
Figure 4.20: Grazing incidence IR of activated monolayer after reaction with rhodamine 110.

Figure 4.21: Grazing incidence IR of activated monolayer after reaction with cresyl violet 670.
Figure 4.22: ATR IR spectrum of solid rhodamine 110.

Figure 4.23: ATR IR spectrum of solid cresyl violet 670.
Figure 4.24: CV analysis of rhodamine 110 covalently coupled to a SAM.

Figure 4.25: CV analysis of cresyl violet 670 covalently coupled to a SAM.

The monolayer was irradiated with a photomask (100 µm x 100 µm squares or 100 µm lines separated by 150 µm), activated with EDC/NHS, and exposed to one of the fluorophores. An amine group of the fluorophore reacts with the activated carboxylic acid and an amide bond is formed on the surface in a pattern. Metals, including gold, quench fluorescence by an energy transfer process,\textsuperscript{151-152} and direct observation of the surfaces by fluorescence microscopy resulted in no patterns being detected. A technique was devised to remove the pattern from the gold surface so that images could be obtained. Transparent Scotch tape was applied to the surface, allowed to incubate at elevated temperature to facilitate transfer, and then removed and adhered to a glass slide for observation. This lift-off technique effectively transferred the fluorescent pattern from the gold surface to eliminate fluorescence quenching by the metal substrate. A diagram of the step-by-step multilayered film assembly process and the tape-transfer method for pattern observation is shown in Figure 4.26.

It is noted that the tape transfer method did not remove the entire film (or entire fluorophore layer as depicted in the diagram) from the surface as patterns were still observed (by wettability differences) after application and removal of tape from the substrate. Also, multiple tape transfer steps on the same substrate resulted in fluorescent patterns still observed; indicating only a portion of the film is removed. Control experiments (described in detail below) coupled with the characterization data for bulk films (described above) demonstrate that the observed patterns are not due to non-specific deposition of the fluorophores on the substrate, but rather indicative of a specifically bound, multilayered film. It is also noted that simply applying a piece of tape to a patterned substrate and removing it resulted in no transfer as no fluorescence pattern was
observed by microscopy. Successful transfer required incubation at elevated temperature in order to remove a portion of the multilayered film for observation.

Patterns were observed with a fluorescence microscope using different excitation and emission filters for each fluorophore, and images of square and line arrays of each fluorophore are shown in Figure 4.27. The tape transfer method results in a distortion of the patterns and contributes micron sized contaminants seen in the images.
Figure 4.27: Fluorescence microscopy images of surfaces patterned with 100 µm squares (top) and 100 µm lines separated by 150 µm spaces (bottom), images on the left are of rhodamine 110 (green) and images on the right are cresyl violet 670 (red).

Inset – reduced scale images of the photomasks used to pattern the surfaces.
Control experiments including the SAM, the irradiated SAM, EDC/NHS activated surfaces, and surfaces exposed to fluorophores with no EDC/NHS activation, imaged using the same methods, result in no patterns being observed, indicating the fluorescent compound is coupled to the surface through an amide bond between the carboxyl group of the film and the amine group of the fluorophore. Patterned surfaces were also treated by sonication in ethanol, washing with dilute acid and base (0.01 M HCl and 0.01 M NaOH), salt (0.1 M AlCl$_3$), and detergent (0.1 M sodium dodecylsulfate, SDS). Patterns are observed after these surface treatments, supporting the formation of a covalently coupled, stable multilayered film.

4.11. Multiple Surface Patterns.

These substrates can be further patterned. By taking the patterned surface, as described above, and re-irradiating after rotating the photomask 45 degrees to the original, a second array of squares is added to the surface. The surface is activated with EDC/NHS and exposed to the fluorophore. Imaging reveals a second array of squares at 45 degrees to the original, as seen in Figure 4.28. These results demonstrate that the multilayered film is stable to further patterning, and that this method is capable of generating films with different areas of functionality.

Patterning experiments were also used to attach multiple fluorophores to the same substrate. A SAM was deposited, deprotected with a square array mask, activated, and exposed to rhodamine 110. The substrate was re-irradiated (with the photomask at 45 degrees to the original), activated, and exposed to cresyl violet 670. Fluorescence microscopy showed a two-colored, dual array on the surface. Observation with a green emission filter reveals the initial pattern of the rhodamine dye, and observation in the same location with a red emission filter shows a
diagonal pattern of the cresyl violet dye, Figure 4.29. It is noted that the rhodamine compound emits light in the wavelength range of the red filter (see Figure 4.19), and is the reason a second dim red square array is seen in the image on the right in Figure 4.29. A combined red/green image of the patterned surface is shown in Figure 4.30. The dual patterned array demonstrates the multilayered approach to surface patterning.

Figure 4.28: Dual square arrays of rhodamine 110 (left) and cresyl violet 670 (right).
Figure 4.29: Dual patterned surface of rhodamine 110 and cresyl violet 670 observed in the same location with a green filter (left) and a red filter (right).

Figure 4.30: Combined red/green image of a dual patterned surface.
CONCLUSIONS

A method for fabricating patterned surfaces has been developed by combining photo-patterning and covalent interactions. Removal of a photolabile portion of a self-assembled monolayer exposes a reactive site, which can be further functionalized by forming amide bonds. This method of multilayered surface patterning is demonstrated by incorporating fluorophores into the assembly structure, allowing for pattern observation and imaging using fluorescence microscopy. Further patterning has shown that the film is stable and that the process can be repeated in other regions of the surface. The multilayered assembly scheme described can generate multi-component surfaces with site-specific areas of chemical functionality with potential utility in biological sensors, directed cell adhesion, switchable surface wettability, and other nanoscale surface applications.
**Future Work**


Photopatterning of optically transparent substrates including glass, polymers, and indium tin oxide (ITO) is useful for many biological applications including directed cell adhesion.\(^{135}\) Microcontact printing has been used to pattern these types of substrates, but limitations including resolution (approximately 5 \(\mu\)m minimum dimensions) and reproducibility of the patterns that are transferred are disadvantages of this technique.\(^{54}\) Photopatterning overcomes these issues because a complete monolayer is formed (reproducibly) in the first step, and patterning by deprotection of a photolabile group occurs in the second step (the resolution is controlled by the photolithographic setup). The resolution for photolithography is sub micron (to \(~300\) nm, depending on the optical setup used) and the increased resolution compared to microcontact printing is advantageous for studies of neuronal axons, which are approximately 2 \(\mu\)m in diameter. Silane chemistry is used to form SAMs on silica and metal oxide substrates,\(^{153-155}\) and incorporation of a photolabile protecting group into the silane SAM facilitates photopatterning.

Two approaches have been identified to pattern optically transparent substrates. The first is to attach a choroisilane with a nitrobenzyl protecting group (2-nitrobenzyl-11-(trichlorosilyl)undecanoate) to a surface. Chlorosilanes polymerize upon standing, and must be prepared immediately prior to surface deposition.\(^{155}\) A stable compound with a free double bond, 2-nitrobenzyl-undec-10-enoate, can be prepared and converted to a chlorosilane immediately prior to SAM deposition, which avoids polymerization. The conversion involves treating the alkene with trichlorosilane in the presence of a catalyst, resulting in addition across
the double bond. Chemical structures of the alkene and chlorosilane are shown in Figures 4.31 and 4.32. Both compounds have been prepared and the synthetic procedures are described in detail at the end of this chapter. Preliminary attempts to deposit and pattern SAMs of the chlorosilane on glass have been unsuccessful (discussed below).

![Chemical structure of 2-nitrobenzyl-undec-10-enoate.](image1)

Figure 4.31: Chemical structure of 2-nitrobenzyl-undec-10-enoate.

![Chemical structure of 2-nitrobenzyl-11-(trichlorosilyl)undecanoate.](image2)

Figure 4.32: Chemical structure of 2-nitrobenzyl-11-(trichlorosilyl)undecanoate.

An alternative approach to generating patterned surfaces using silane chemistry involves multilayered films formed by covalent surface reactions. The multilayered film is assembled on the substrate by first using aminopropyltriethoxysilane (forming an amine terminated surface),
and then exposing the monolayer to a compound with a carboxyl group to form an amide bond.\textsuperscript{112-113} Carbodiimide chemistry activates the carboxylic acid, analogous to the multilayered film assembly strategy described previously. Aminopropyltriethoxysilane has been used as a base layer on glass, or other substrates such as quartz or ITO, for multilayered films.\textsuperscript{30} A compound with a nitrobenzyl protecting group and a free carboxylic acid, 11-(2-nitrobenzyloxy)-11-oxoundecanoic acid, has been identified and synthesized. The chemical structure of this compound is shown in Figure 4.33, and a synthetic procedure is described in detail at the end of this chapter. As with the trichlorosilane, preliminary attempts pattern surfaces using this compound were unsuccessful.

![Figure 4.33: Structure of 11-(2-nitrobenzyloxy)-11-oxoundecanoic acid.](image)

SAM deposition conditions will be optimized for this system. Silanes are not dynamic on a surface like thiols are on gold, and forming highly ordered films that can be further patterned is not analogous.\textsuperscript{155} Variations in deposition conditions including solvent, silane concentration, incubation time, temperature, as well as methods for substrate preparation will be investigated. Exposure of piranha cleaned glass substrates to a 5 mM solution of the trichlorosilane in toluene for 18 hours resulted in sessile drop contact angles of 70° to 80°, similar to those obtained for SAMs on gold. Irradiation with 300 nm light for two hours resulted in negligible changes in the
contact angle (a hydrophilic surface was not generated) that would be observed if a carboxylic acid terminus was present. Experiments where SAMs were deposited from both toluene and hexadecane for periods between 1 and 6 hours (1 hour intervals) also resulted in substrates that showed no change in contact angle following irradiation. No patterns were observed (based on wettability differences) when irradiating these substrates through a $100 \times 100 \, \mu\text{m}$ square array photomask. It is unclear whether the lack of change in wettability is due to the monolayer deposition step or the deprotection step. Future work includes testing different deposition conditions, as described above, and then further characterizing the monolayer by XPS in addition to contact angle measurements, to confirm SAM formation prior to deprotection.

4.13. Photoprotected Amine Terminated Surface.

The ability to generate reactive surface groups in patterns on a substrate allows multi-component films to be assembled in specific regions. This chapter discusses using photolabile protecting groups to prepare carboxyl terminated functionalities that can be further modified through covalent coupling. A method that forms patterned amine functionalities on a substrate is of interest for further surface modification. Amine terminated surfaces are used as a base layer for multilayered film deposition through the formation of amide bonds to moieties with free carboxyl groups that are first activated in solution.\textsuperscript{112-113} Several reports have detailed using nitroveratryloxy carbonyl (NVOC) protecting groups to generate different types of amine reactive surface sites that can be further functionalized.\textsuperscript{134-136} NVOC protecting groups are used in organic synthesis as a way to generate free amines by photocleavage using ultraviolet light (350 nm).\textsuperscript{143-144} NVOC protecting groups undergo Norrish-type II photoreactions converting the $\alpha$-nitrobenzyl alcohol derivative into an $\alpha$-nitrosobenzaldehyde, the mechanism of which is
described in section 4.3. Although these reports have demonstrated successful surface patterning, a method for generating reactive amine sites on gold followed by coupling to free carboxyl groups in specific patterns has not been described.

A compound with a NVOC protected amine group and a thiol linker has been identified. The structure of this compound, 4,5-dimethoxy-2-nitrobenzyl-10-mercaptopdecylcarbamate is shown in Figure 4.34. After SAM deposition, irradiation and removal of the protecting group will reveal an amine group capable of forming an amide bond with an activated carboxylic acid, and result in a multilayered film. Future work includes synthesizing the target compound, depositing the target as a SAM, characterizing the film, and irradiation and removal of the protecting group.

![Figure 4.34: Structure of 4,5-dimethoxy-2-nitrobenzyl-10-mercaptopdecylcarbamate.](image)


Modified substrates have been investigated for studies of controlled cell shape, position, function, and proliferation. Directed nerve cell growth on surfaces is of interest in constructing neuronal networks. Self-assembled monolayers with photolabile
protecting groups can be used to generate patterned surfaces for cell adhesion. Photopatterned SAMs can be fabricated with specific areas that promote cell adhesion and growth, and other areas that inhibit cell attachment. A multi-step process using a photolabile SAM that leaves a reactive surface site after irradiation allows for cell promoting regions to be added to the substrate in the deprotected areas, and can also be used to add multiple functionalities following subsequent deprotection steps.

Preliminary work has demonstrated patterned neuronal cell growth using this method. A SAM of a photolabile compound (2-nitrobenzyl-11-mercaptoundecanoate) was deposited on a gold substrate, and deprotected in a pattern of 100 µm lines separated by 150 µm spaces. The carboxylic acid group was activated (as described previously) and the surface was exposed to ethylenediamine to convert the carboxylic acid terminated surface to an amine terminated surface (experiments have demonstrated that neuronal cells preferentially adhere to amine surfaces). The patterned substrates were incubated with nerve cells using the Neuroscreen 1 cell line, cultured in RPMI medium (developed by Moore et al. at Roswell Park Memorial Institute) with 15 % serum, and differentiated with nerve growth factor (NGF). After five days of incubation, images were obtained that showed the cells adhering and growing in a pattern on the substrate (Figure 4.35). The cells preferred to grow on the portion of the substrate that had been deprotected and converted to an amine termination (based on the size of the pattern); this was confirmed by experiments using substrates that were either completely protected or deprotected (no pattern used).
Future work includes determining the optimum surface chemistry for cell attachment; amine terminal groups were selected for this preliminary experiment because neurons have been shown to preferentially adhere to amine substrates. Other surfaces chemistries, such as polylysine, have also been shown to promote nerve cell adhesion, and a comprehensive study of substrates patterned with different functionalities (NH$_2$, COOH, OH, CH$_3$, etc.) will determine the optimum surface chemistry for this cell line (Neuroscreen 1). Different surface chemistries are investigated by patterning the SAM, incubating with neuronal cells, and monitoring cell growth by taking images of the substrate each day. Comparisons between substrates are made based on the number of cells attached to the surface and how well defined the patterns of cells are. A second deprotection step, followed by converting the remainder of the substrate to a functionality that inhibits cell attachment, will improve patterning. For example, substrates coated with
polyethylene glycol (PEG) inhibit cell adhesion, and a substrate that contains both adhesive (amine) and resistive areas (PEG) will direct nerve cell growth in a pattern.

4.15. Non-Covalent Patterning.

As previously described in section 4.7, multilayered patterning using non-covalent interactions was investigated. A diagram of the multilayered assembly process is shown in Figure 4.36. A gold substrate (1) is modified with a SAM of 2-nitrobenzyl-11-undecanoate (2), which is irradiated through a photomask (3) resulting in a patterned substrate that has both protected and deprotected areas (4). The deprotected carboxylic acid portion forms a complex with metal ions (5) and is ‘capped’ with another organic ligand (6). The unirradiated areas can be further patterned. In order for this assembly process to be feasible, the metal ions must complex with the deprotected regions and not interact with the unpatterned areas of the substrate (step 5 in Figure 4.36).

Characterization results for SAMs of 2-nitrobenzyl-11-mercaptopoundecanoate (unirradiated) demonstrated that metal ion exposure produced irreversible changes in the films that were not consistent from one metal ion to another. For example, Cu(II) exposure resulted in the electrochemical behavior of the film changing from blocking to non-blocking to a redox process in solution (ferricyanide), and patterned SAMs incubated in a Cu(II) solution for times as short as 1 minute were no longer patterned based on wettability differences. Exposing unirradiated SAMs to Pb(IV) ions caused no changes in the blocking nature of the film when examined by CV, but XPS analysis showed Pb ions present in the film. A list of all metal ions investigated is provided in section 4.7. The type of interaction between metal ions and the protected regions of
the surface was not determined, and no correlation between metal ion oxidation state or ionic radius and film properties was identified. It may be a specific interaction such as complexation with either the nitro group or carbonyl of the ester (or a combination of the two), or a non-specific deposition or penetration of the metal ion into the film. In either case, future work includes determining the type of interaction in order to fabricate multilayered films non-covalently.

Figure 4.36: Non-covalent multilayered assembly process.
High resolution XPS analysis provides information that cannot be determined from contact angle, IR, and CV measurements.\textsuperscript{41} This information includes the overall chemical composition of the film, the oxidation state of the metal, and the bonding occurring in the system. For example if the metal ion is forming a specific interaction with the carbonyl of the protected SAM, changes in the binding energy of the carbon and oxygen atoms present in the film will be observed. Analysis of high resolution spectra will allow for these changes to be quantified and provide a model for the bonding interactions in the film. A comprehensive examination of different metal ions (such as those described in section 4.7) exposed to both protected and deprotected SAMs by XPS may determine if certain metal ions can be used for multilayered assembly with this system, and also discern why specific metal ions cause the different changes in the film described above.
EXPERIMENTAL DETAILS

Materials.

All chemicals and solvents were reagent grade or better, from Aldrich (Milwaukee, WI) or Alfa Aesar (Ward Hill, MA), and used as received unless otherwise noted. Rhodamine 110 from Acros Organics (Geel, Belgium). Cresyl violet 670 from Exciton (Dayton, OH). Ethanol, 200 proof, absolute, for all experiments from Pharmco Products (Brookfield, CT). Silica gel, 40 μm, 60 Å, for column chromatography from J.T. Baker (Phillipsburg, NJ). De-ionized water from a Millipore (Billerica, MA) Synergy UV system.

Instrumental Analysis.

NMR spectra were obtained on either a Bruker (Billerica, MA) Avance 400 MHz spectrometer or a Bruker Avance III 500 MHz spectrometer and referenced to tetramethylsilane (TMS). Spectra were recorded at 400 MHz or 500 MHz for $^1$H and 100 MHz or 125 MHz for $^{13}$C, and all chemical shifts (δ) are reported in ppm. Mass spectrometry was performed on a Waters (Milford, MA) Micromass model ZMD spectrometer using electrospray ionization and a 50:50 acetonitrile:water solvent flow. Attenuated total reflectance (ATR) infrared spectroscopy experiments were carried out on a Thermo Scientific (Waltham, MA) Nicolet FT-IR model 6700 spectrometer using a liquid nitrogen cooled, mercury cadmium telluride (MCT) detector. UV/Visible spectra were recorded on a Perkin Elmer (Wellesley, MA) Lambda 35 UV/Vis double beam spectrometer, and a baseline correction (solvent vs. solvent) was performed prior to each experiment. Fluorescence measurements were performed on a Perkin Elmer model LS50B Luminescence Spectrometer. Transmission (bright-field) optical microscopy was performed on
a Fisher (Pittsburg, PA) Micromaster optical microscope with an integrated digital camera coupled to a computer running Micron (v 1.01) software.

*Preparation of Self-Assembled Monolayers.*

Gold surfaces were obtained commercially from Evaporated Metal Films (EMF) (Ithaca, NY). The float glass slides (25 mm x 75 mm x 1 mm) are coated with 50 Å of a chromium adhesion layer followed by 1000 Å of gold. Prior to monolayer formation, the slides were cut to size (2 cm x 1 cm for most experiments) and cleaned by immersion in a piranha solution (70 % concentrated sulfuric acid, 30 % concentrated hydrogen peroxide) at 90 °C for 10 minutes. The slides were then washed thoroughly with distilled water, followed by absolute ethanol, and then dried in a stream of nitrogen. The cleaned slides were immediately placed in the monolayer solution (5 mM in ethanol) overnight. After deposition, and prior to any characterization, the films were removed from solution, rinsed with ethanol, and dried with nitrogen. New films were prepared immediately prior to characterization.

*Photo-Deprotection.*

Removal of the photo-labile protecting group was accomplished by exposing the slides to ultraviolet light in a Rayonet reactor. The lamp used was a 300 nm mercury arc lamp. The slides were placed in deionized water and irradiated for 2 hours. For photomask experiments the irradiation was accomplished by clamping the photomask to the substrate using a custom built clamping fixture. No solvent was used for patterning experiments. After irradiation the slides were rinsed with deionized water, followed by ethanol, and dried under nitrogen.
Photomasks.

Custom fabricated photomasks were obtained from Adtek Photomask (Montreal, Canada). The specified design and dimensions were printed on soda lime glass (3” x 3” x 0.06”) using a chrome coating. The masks were rinsed thoroughly with distilled water and ethanol after each use.

Surface Activation and Multilayered Film Assembly.

Irradiated substrates were exposed to a freshly prepared solution of 0.1 M EDC and 0.02 M NHS in deionized water for 30 minutes while agitating with a Thermo Scientific (Waltham, MA) Barnstead/Lab-line Lab Rotator on low speed to facilitate the reaction. Following activation, the substrates were rinsed with deionized water, dried with nitrogen, and placed in a 0.01 M ethanolic solution of a fluorescent compound (rhodamine 110 or cresyl violet 670) for 10 minutes (with agitation) to complete the surface reaction. The samples were thoroughly rinsed with ethanol and dried with nitrogen. Patterned surfaces were shielded from light using aluminum foil during the activation and multilayered film assembly process.

Fluorescence Microscopy.

Patterned surfaces were examined by fluorescence microscopy. Fluorescent images were obtained on a Nikon (Melville, NY) Eclipse model E600 fluorescence microscope equipped with a Diagnostic Instruments (Sterling Heights, MI) RT Color digital camera and Spot (v. 4.0) analysis software. Samples were observed using a Nikon Plan Fluor 10 x (N = 0.50) Ph1 DLL lens. Illumination was provided by a mercury arc lamp (100 W, Chiu Technical Corporation, Kings Park, NY) passed through either a Nikon FITC–HYQ (excitation filter: 460–500 nm,
dichroic mirror cut on: 505 nm, barrier filter: 510–560 nm) filter cube for green fluorescence or a Nikon Texas Red HQ (excitation filter: 532–587 nm, dichroic mirror cut on: 595 nm, barrier filter: 608–683 nm) filter cube for red fluorescence. Patterned surfaces were prepared for microscopy analysis by removing the pattern from the gold slide with tape. Slides were coated with strips of Scotch brand transparent tape from 3M (St. Paul, MN) and incubated at 80 °C for 5 minutes to facilitate pattern transfer. The tape was carefully removed from the surface and adhered to a standard glass microscope slide for analysis and imaging.
**SYNTHETIC DETAILS**

2–nitrobenzyl–11–mercaptoundecanoate (photolabile compound).

One equivalent of 2–nitrobenzyl alcohol (2.17 g, 14.15 mmol) was combined with one equivalent of 11-mercaptoundecanoic acid (3.09g, 14.15 mmol), 0.1 equivalents DMAP (0.173 g, 1.42 mmol) and dissolved in dichloromethane (50 mL). To this mixture a solution of DCC (2.92 g, 14.15 mmol) in dichloromethane (30 mL) was slowly added while stirring. Upon addition, a white precipitate formed after a few minutes. The mixture was stirred at room temperature overnight. Vacuum filtration removed the white precipitate and the filtrate was concentrated under vacuum. Silica gel column chromatography was used for purification with dichloromethane/methanol (v/v 50:1) as eluent. The product was dried over sodium sulfate, the solvent removed by rotary evaporation and dried under vacuum, producing a yellow solid. Yield: 3.57 g (71 %). $^1$H–NMR (CDCl$_3$) $\delta$(ppm): 8.1 (d, 1H, Ar), 7.6 (t, 1H, Ar), 7.5 (d, 1H, Ar), 7.4 (t, 1H, Ar), 5.5 (s, 2H, O–CH$_2$), 2.6 (t, 1H, SH) 2.5 (q, 2H, CH$_2$–SH), 2.4 (t, 2H, CH$_2$–C=O), 1.6 (m, 4H, CH$_2$), 1.2 (m, 12H, CH$_2$). $^{13}$C–NMR (CDCl$_3$) $\delta$(ppm): 173.5, 147.0, 134.1, 132.7, 129.5, 129.2, 125.5, 63.2, 34.6, 34.4, 29.8, 29.7, 29.6, 29.5, 29.4, 28.8, 25.3, 25.1. MS (ESI): (M + Na)$^+$ = 376.19 (calc. 376.47).
2-Nitrobenzyl-undec-10-enoate.

One equivalent of 2-nitrobenzyl alcohol (2.39 g, 15.65 mmol) was combined with one equivalent of undecylenic acid (2.88 g, 15.65 mmol) and 0.1 equivalents of DMAP and dissolved in 50 mL CH₂Cl₂. DCC (3.23 g, 15.65 mmol) was dissolved in 30 mL dichloromethane and slowly added to the mixture while stirring. A white precipitate slowly formed after about 5 minutes. The reaction mixture was stirred at room temperature overnight. The precipitate was filtered off by vacuum filtration and the filtrate was concentrated by rotary evaporation. Silica gel column chromatography with dichloromethane/methanol (v/v 50:1) as eluent was used for purification. The product was dried over sodium sulfate, solvent removed by rotary evaporation, and dried under vacuum producing a yellow oil. Yield: 3.91 g (78.2 %). $^1$H-NMR (CDCl₃) δ(ppm): 8.1 (d, 1H, Ar), 7.7 (t, 1H, Ar), 7.6 (d, 1H, Ar), 7.5 (t, 1H, Ar), 5.8 (m, 1H, CH=CH₂), 5.5 (s, 2H, CH₂-O) 4.9 (dd, 2H, CH₂=CH), 2.4 (t, 2H, CH₂-C=O), 2.0 (q, 2H, CH₂), 1.6 (m, 2H, CH₂), 1.2 (m, 10H, CH₂). $^{13}$C NMR (CDCl₃) δ (ppm): 173.5, 147.9, 139.5, 134.1, 132.7, 129.4, 129.1, 125.4, 114.6, 63.2, 34.5, 34.2, 29.7, 29.6, 29.5, 29.4, 29.3, 25.3. MS (ESI): (M + H)$^+$ = 320.30 (calc. 320.40).
2-Nitrobenzyl-(11-trichlorosilyl)-undecanoate.

Previously prepared 2-nitrobenzyl undec-10-enoate (1.32 g, 4.14 mmol) was placed in a flask and excess trichlorosilane (8.36 mL, 82.8 mmol) was added under nitrogen protection. A 0.8 M solution of hydrogen hexachloroplatinate (IV) hydrate in 2-propanol was prepared and 60 μL of this solution was added to the reaction mixture while stirring. The mixture was stirred at room temperature overnight. The excess trichlorosilane was removed by vacuum, leaving a yellow oil. Yield: 1.40 g (74.5%). $^1$H-NMR (CDCl$_3$) δ (ppm): 8.1 (d, 1H, Ar), 7.7 (t, 1H, Ar), 7.6 (d, 1H, Ar), 7.5 (t, 1H, Ar), 5.5 (s, 2H, CH$_2$-O), 2.4 (t, 2H, CH$_2$-C=O), 1.6 (m, 4H, CH$_2$), 1.2 (m, 14H, CH$_2$). $^{13}$C NMR (CDCl$_3$) δ (ppm): 173.5, 147.9, 134.1, 132.7, 129.4, 129.1, 125.4, 63.2, 34.5, 32.2, 29.9, 29.7, 29.6, 29.5, 29.4, 29.3, 24.7, 22.6.
**11-(2-Nitrobenzylxyloxy)-11-oxoundecanoic acid.**

2-nitrobenzyl alcohol (0.708 g, 4.6 mmol), undecanedioic acid (1.0 g, 4.6 mmol), and DMAP (0.056 g, 0.46 mmol) were combined in a flask with 20 mL dichloromethane. To this mixture a solution of DCC (0.954 g, 4.6 mmol) in 10 mL dichloromethane was slowly added. The reaction was stirred overnight at room temperature and a white precipitate formed. The precipitate was removed by vacuum filtration and the filtrate was concentrated under vacuum. Silica gel column chromatography with dichloromethane/methanol (v/v 50:1) as eluent was used to purify the product. The product was dried over sodium sulfate, the solvent removed by rotary evaporation, and the solid dried under vacuum, affording a white powder. Yield: 0.488 g (30 %). \(^1\)H-NMR (CDCl\(_3\)) \(\delta (ppm)\): 11.1 (s, 1H, OH), 8.1 (d, 1H, Ar), 7.6 (d, 1H, Ar), 7.5 (t, 1H, Ar), 7.4 (t, 1H, Ar), 5.5 (s, 2H, O-CH\(_2\)), 2.3 (m, 4H, CH\(_2\)), 1.6 (m, 4H, CH\(_2\)), 1.2 (m, 10 H, CH\(_2\)). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta (ppm)\): 178.9, 173.5, 157.5, 134.0, 132.7, 129.4, 129.1, 125.4, 63.1, 34.5, 34.3, 34.1, 29.6, 29.5, 29.4, 25.9, 25.2, 25.1. MS (ESI): (M + Na)\(^+\) = 374.23 (calc. 374.38).
5: **Switchable Wettability**
INTRODUCTION

Modified surfaces with properties that can be regulated in response to external stimuli such as changes in light, solvent, pH, temperature, or electric potential is of interest for areas such as: information storage, microfluidics, biosensing, and other fields.\textsuperscript{166-177} Ralston \textit{et al.} showed that SAMs of long-chain thymine-terminated thiols exhibited a contact angle change of $26^\circ$ after irradiation with UV light.\textsuperscript{178} Liu \textit{et al.} reported reversible conformational behavior of 16-mercaptophexadecanoic acid under positive and negative applied potentials, which resulted in a change in wettability.\textsuperscript{179}

Systems that undergo reversible photoinduced wettability changes are of particular interest as reversible gates in microfluidic applications such as microreactors. In effect, the photolabile monolayer described in Chapter 4 is an irreversible wettability switch. Irradiation and removal of the protecting group results in a decrease in surface wettability that could be used to open a fluidic “gate” but because the change is irreversible, the gate could not be closed. Development of a reversible system using a multilayered film would allow for a gate to be placed in a specific region of a fluidic device, with light being used as the stimulus to open and close the gate. Fluidic gates in microreactors would allow for small amounts of liquid to be selectively introduced into a reaction chamber at precise volumes and intervals. Microreactors or “lab-on-a-chip” systems have advantages that include small amounts of reactants and solvents that minimize waste produced, reactions can be carried out at a small scale if quantities of starting materials are limited, and that many different reactions can be carried out in a small system.
Previous work includes fabrication of stable, non-covalently bound multilayered films on gold surfaces using 4-(10-mercapto-decyloxy)-pyridine-2,6-dicarboxylic acid as the SAM. The pyridine head group of this SAM was used as a ligand to bind a layer of copper ions [Cu(II)], which was subsequently complexed to another organic ligand. This non-covalent approach was used to fabricate films that exhibited photo-switchable wettability. A capping ligand of cis-2,2’-dipyridylethylene produced a hydrophobic surface, and UV irradiation resulted in a decrease in hydrophobicity (19º reduction in contact angle). Spectroscopic studies indicated that this reduction in contact angle was due to cis- to trans- photoisomerization of the capping pyridylethylene ligand. For this system, the isomerization and therefore the change in surface wettability were irreversible, thus limiting its utility for most applications.

Irreversible cis-/trans- photoisomerization on surfaces has been reported previously. Fox et al. showed that a trans-stilbene isomer could not be converted to cis- when the compound was deposited as a SAM, which is believed to be due to organization of the trans- ligand on the surface creating a steric barrier to the trans- to cis- isomerization process. In effect, the trans-isomer self-assembles on the surface in an orientation that provides insufficient space to allow isomerization to the cis- conformation.

The goal of the work described in this chapter is three-fold. The first step is to fabricate and characterize a multilayered film utilizing non-covalent assembly with a terminal group capable of undergoing reversible isomerization when exposed to light. The photoswitching ligands used in these studies were cis- and trans- stilbene-4,4’dicarboxylic acid, as stilbene compounds undergo reversible cis-/trans- isomerization upon exposure to ultraviolet light. The
second aim is to demonstrate that the isomerization of the stilbene moiety is reversible and results in a change in surface wettability. The final step is to optimize the surface spacing between the stilbene isomers in order to increase the yield of the isomerization process. Two methods were used to control surface spacing of the binding sites: mixed monolayers and photolabile protecting groups. The mixed monolayer approach uses short chain t-butyl benzene groups to space out the dicarboxypyridine binding sites. SAMs with photolabile moieties were also used to generate binding sites with increased spacing after irradiation and cleavage of the protecting group. This chapter describes these methods for binding site separation and the resulting reversibility of the photoisomerization process as evidenced by changes in surface wettability.
RESULTS AND DISCUSSION

5.1. Multilayered Film Fabrication and Characterization.

Multilayered thin films containing 4,4’-stilbene-dicarboxylic acid were fabricated by sequential deposition of individual layers that are bound by non-covalent, metal-ligand interactions, as shown in Scheme 5.1. These films consist of a SAM made up of 4-(10-mercapto-decyloxy)-pyridine-2,6-dicarboxylic acid deposited on a gold surface which acts as a ligand for binding Cu(II) ions.\textsuperscript{182-183} The metal ion was then ‘capped’ by deposition of each stilbene isomer. While it is not possible to unambiguously identify the binding mechanism, it is likely that binding occurs between the Cu(II) ion and one of the carboxylic acid groups of the stilbene. Binding between carboxylic acid groups and Cu(II) ions in self-assembled films has been reported previously.\textsuperscript{119-120,184}

The selection of 4,4’-stilbene-dicarboxylic acid as the capping ligand was made for three reasons. First, the cis- and trans- isomers of this compound were shown to undergo reversible photoinduced isomerization in solution based on UV/Vis analysis.\textsuperscript{185} Secondly, the carboxylic acid group acts as a metal-binding ligand and thereby anchor the compound to the film. Finally, isomerization will change the orientation of the non-bound carboxylic acid group relative to the surface and as a result alter the wettability.

Although Scheme 5.1 is an idealized representation, the stilbene isomers will provide different surface properties when bound to a substrate if the films assemble in this manner. The \textit{cis}-stilbene-4,4’-dicarboxylic acid will create a hydrophobic surface when bound to a metal ion
通过一个羧基基团（CH基团暴露）。相反，转-形式的化合物将生成一个疏水表面，当非共价连接到一层通过一端分子（COOH基团暴露）时。Cis-trans异构化将重新定向末端基团并改变表面润湿性。

**Scheme 5.1: Fabrication of the multilayered film.**

![Scheme 5.1: Fabrication of the multilayered film.](image)

在每层（SAM, Cu(II), 和 stilbene）沉积后，通过接触角测定法来表征这些薄膜以确定润湿性，通过漫反射IR来证明化学性。

Following deposition of each layer (SAM, Cu(II), and stilbene) the films were characterized by contact angle goniometry to determine wettability, grazing incidence IR to demonstrate chemical
functionality, and cyclic voltammetry to estimate surface coverage. The results of these characterization studies indicate that the underlying SAM forms a nearly complete layer. This is supported by characteristic IR bands at 2922 cm\(^{-1}\) and 2852 cm\(^{-1}\), which indicate that the SAM is well ordered.\textsuperscript{140,186-187} Cyclic voltammetry measurements conducted with the film on gold as the working electrode, using a redox probe show blocking behavior.\textsuperscript{84}

After metal ion complexation, stilbene isomers were deposited on the surface. The cis- isomer results in a sessile water droplet contact angle of 76° ± 2.0° and the trans- isomer gives a contact angle of 50° ± 2.0°. These results confirm that the orientation of the cis- isomer on the surface is such that the un-bound carboxylic acid group is not exposed, but rather buried in the film. Conversely, the lower contact angle for the trans- isomer indicates that the un-bound carboxylic acid group is exposed, resulting in a more hydrophilic surface. Grazing incidence IR spectra of stilbene-capped films show the C=C stretch of the stilbene is at approximately 1680 cm\(^{-1}\), which is consistent with that observed in the solid compound. The IR spectra for each isomer are indistinguishable.

The results reported above support the assumption that when irradiated at the appropriate wavelengths the cis- and trans- stilbene capped films will undergo isomerization and that the contact angle, IR and cyclic voltammetry measurements will confirm that this process occurs.

5.2. \textit{UV-Irradiation of Multilayered Films.}

Irradiation of multilayered films of the cis- and trans- stilbene at 254 nm and 350 nm (based on the maximum stilbene absorption band from UV/Vis measurements) respectively, did not result
in reversible wettability changes. Although there was a change in contact angle of the surfaces upon irradiation, the resulting contact angle of each isomer does not match with that of the unirradiated film of the other isomer. The C=C singal at 1682 cm^{-1} in the IR spectra was absent in both irradiated films. The lack of an IR C=C stretching vibration after irradiation suggests that the capping stilbene moiety was either removed from the surface or underwent photodimerization upon exposure to UV light.

This behavior is similar to other previously published studies. For example, UV irradiation of films composed of azobenzene derivatized alkanethiols leads to photodegradation and an associated reduction in water droplet contact angles.\textsuperscript{175} Fox et al. showed that SAMs consisting of cis- and trans- 4-cyano-4’-(10-(acetylthio)deoxy)stilbene do not photo-isomerize, but instead undergo dimerization (2 + 2 cycloaddition). This behavior was attributed to steric barriers to isomerization that result when the SAMs form well-ordered and tightly-packed films.\textsuperscript{181} Given that the C=C stretching vibration is absent following irradiation in the multilayered films, it is likely that a photodimerization reaction has occurred forming a four membered ring between neighboring stilbene moieties. To further examine this possibility, mixed films were used to separate the isomers so that photodimerization on the surface is not possible due to the increased distance between neighboring stilbenes.

5.3. Preparation and Irradiation of Films Fabricated with Mixed Monolayers.

Previous studies have investigated methods to create ample spacing on substrates for conformational transformations. To alleviate steric crowding and facilitate isomerization, Hu et al.\textsuperscript{181} used colloidal gold clusters instead of planar gold surfaces. Film packing on gold clusters
is not as ordered as on a planar gold surface and therefore does not lead to steric concerns. Mixed or two-component monolayers have also been used to create separation in monolayer films, as well as for other applications.

To test the effect of alleviating steric crowding in the multilayered films examined in this study, a mixed SAM of 4-(10-mercapto-decyloxy)-pyridine-2,6-dicarboxylic acid and 4-tert-butylbenzenethiol was deposited. The films were deposited from a solution containing an equimolar mixture of the two thiols (experiments were conducted with different monolayer ratios to determine optimum conditions). The coadsorption of thiols is the general method for generating mixed monolayer films that have different chain lengths or different terminating functional groups. However, factors governing the formation of mixed monolayers are not well understood. Mixed monolayers have been shown to form islands of like molecules on the surface, which limits the ability to uniformly deposit the metal and the capping ligand.

The successful assembly of these mixed monolayers was confirmed by contact angle, grazing IR, and electrochemical measurements as for the previously described films. Irradiation of the cis-mixed monolayer at 254 nm resulted in a change decrease in contact angle of ~ 25° (as shown in Table 5.1) indicating a change from a hydrophobic surface to a more hydrophilic surface. IR spectra obtained before and after irradiation show the stilbene moiety is present following irradiation; a C=C stretching vibration at 1680 cm\(^{-1}\) is observed in both spectra. It is noted that the observed decrease in the contact angle (~ 25°) is substantially greater than that reported for photoisomerizable thin films of azobenzenes or spiropyrans that exhibit changes of ~ 9°.
The \textit{trans}- mixed monolayer film exhibits analogous behavior. Table 5.1 also shows the contact angles obtained for the \textit{trans}- film before irradiation, after irradiation at 350 nm, and after further irradiation at 254 nm. The contact angle obtained after 350 nm irradiation is similar to that of the unirradiated \textit{cis}- mixed monolayer film (65° vs. 70°), and after re-irradiating at 254 nm returns to close to the initial value (48° vs. 45°). IR data also shows the C=C stretching is observed at 1680 cm\(^{-1}\) following irradiation, supporting the conclusion that the stilbene is undergoing photoreversible isomerization in the film.

Further irradiation of either of these mixed monolayer films beyond a single cycle did not result in reversion to the original contact angles. Instead, further irradiation of both films resulted in a convergence of the contact angles to a value intermediate between those measured for the individual isomers. The convergence of contact angles to intermediate values between those of the initial films is indicative of a mixture of \textit{cis}- and \textit{trans}- isomers on the surface, each photoirradiation step has not proceeded completely and a disorganized arrangement results with steric barriers to further isomerization. Mixed monolayers have improved the reversibility of this system over that of uniform monolayers, however the reversibility remains limited. Other methods of binding site spacing were investigated to develop multilayered films with increased reversibility for wettability changes.
Table 5.1: Contact angle measurements for mixed monolayer films.

<table>
<thead>
<tr>
<th>Film</th>
<th>Contact Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed Monolayer + Cis-Stilbene</td>
<td>70° ± 2.0°</td>
</tr>
<tr>
<td>Irradiation at 254 nm</td>
<td>45° ± 2.0°</td>
</tr>
<tr>
<td>Second irradiation at 350 nm</td>
<td>66° ± 1.0°</td>
</tr>
<tr>
<td>Mixed Monolayer + Trans-Stilbene</td>
<td>45° ± 2.0°</td>
</tr>
<tr>
<td>Irradiation at 350 nm</td>
<td>65° ± 2.0°</td>
</tr>
<tr>
<td>Second irradiation at 254 nm</td>
<td>48° ± 1.0°</td>
</tr>
</tbody>
</table>

5.4. Preparation and Irradiation of Films Fabricated with Photolabile Monolayers.

Mixed monolayers can be used to alleviate steric crowding. However, it is difficult to control the spatial distribution of thiols on the surface (e.g., it is not possible to ensure that the monolayers are homogeneous). In order to create a homogenous surface as well as one that provides sufficient separation for isomerization to take place, multilayered films based on a SAM that contains a bulky photolabile head group, 2-nitrobenzyl-11-mercaptoundecanoate, were used.

Langer et al. have demonstrated that bulky head groups can be used to generate spacing between monolayer chains as evidenced by electrochemical switching of the alkyl chain conformation following removal of the head group. This result indicates that even though SAMs of thiols on gold are dynamic on the surface, removal of the head group does provide the effect of increasing the overall distance between alkanethiol chains compared to a conventionally prepared monolayer. It is proposed that after the terminal groups are removed, the metal ion
complexation sites will be sufficiently separated for photoisomerization to occur once the multilayered film has been assembled. Creating surface space by utilizing photolabile monolayers is advantageous over using mixed monolayers because the deprotection will provide a uniform surface with the metal ion complexation sites having equal spacing.

Reorganization of the SAM following irradiation to a densely packed state is a possibility, as the inter chain van der Walls interactions drive the self-assembly process. A reorganization of the alkyl chains would limit the ability of the system to generate separation between binding sites and also result in areas of no monolayer coverage as the chains re-orient closer together. However, the CV characterization data (discussed in Chapter 4) shows that redox current is attenuated for both the monolayer and irradiated monolayer. If the irradiated monolayer re-organized significantly an increase in current for the redox process would be expected as areas of the substrate with no monolayer coverage would not block electron transfer. Observation of similar attenuated current to the SAM indicates that reorganization of the film is minimal in that is does not result in areas of the substrate with no monolayer coverage, and supports the assumption that bulky protecting groups can be used to separate reactive surface sites.

A comprehensive description of SAM deposition, deprotection, and characterization for the photolabile compound is provided in the previous chapter. Following deprotection, the carboxylic acid terminated SAM was exposed to Cu(II) ions and then cis-stilbene-4,4'-dicarboxylic acid to complete the multilayered film. The multilayered assembly process is depicted in Scheme 5.2. Attempts to deposit the trans- isomer in the same manner were unsuccessful. Although grazing incidence IR showed the presence of the stilbene moiety on the
surface, the contact angle was surprisingly high (~ 90°), which is unexpected if a terminal carboxylic acid group is present. The high contact angle indicates that another portion of the compound is exposed to the air interface, and this observation is likely due to the increased separation between binding sites not providing the trans-stilbene isomer a suitable template for self-assembly. Irradiation of the trans-stilbene exposed substrate did not produce a change in contact angle, further supporting this assumption.

**Scheme 5.2:** Fabrication of multilayered films using photolabile monolayers.
Contact angle, grazing IR, and CV analysis were performed on the multilayered film. The contact angle of the cis-stilbene capped film was 64° ± 2.0°, similar to the other cis-stilbene terminated surfaces discussed above. Grazing incidence IR measurements showed characteristic absorptions of the stilbene compound (results discussed below). Electrochemical measurements of the multilayered film were analogous to that of the deprotected SAM exposed to Cu(II) ions, which is also identical to bare gold. Although some multilayered systems do show blocking behavior when examined by CV, the lack of blocking behavior is not necessarily indicative of a poorly ordered or non-existent film. The protected and deprotected photolabile monolayers exhibit blocking behavior to a redox process in solution (results presented and discussed in Chapter 4), however upon exposure to metal ions, the CV profile of the film matches that of bare gold. This observation may be due to the film becoming ‘leaky’ when a metal ion complexes to the terminal carboxylic acid group. The interaction with the metal ion causes a reorganization of the film that allows electron penetration to the gold surface, and the solution redox process is observed by CV measurements.

An alternative explanation is that when a positively charged metal ion is bound to the surface, the electrostatic interaction between the film and the negatively charged redox probe is increased, resulting in a non-blocking layer. Similar results are obtained when comparing monolayers of mercaptoundecanoic acid (negatively charged) and aminoundecanethiol (positively charged). The carboxylic acid terminated surface is blocking to the redox process of ferricyanide, while the amine terminated surface is not. In either case, the addition of the stilbene isomer to the film does not cause a reversion to blocking behavior. This observation may be due to increased separation of the capping layer as compared to the mixed monolayer
system described above, which would be advantageous for the reversibility of the photoisomerization process. CV analysis is therefore not particularly useful for this multilayered system, and better evidence of the assembly process is provided by the contact angle and IR data.

Films terminated with the cis-stilbene isomer were irradiated repeatedly at alternating wavelengths (254 nm and 350 nm). The choice of wavelength was based on the absorbance spectra of these compounds, which are provided in Figure 5.1. Contact angles were measured after each irradiation step and the results are summarized in Table 5.2. The results were similar to those obtained for films prepared from mixed monolayers in that irradiation results in isomerization based on the contact angle measurements. The change in contact angle was ~ 20°, and subsequent irradiation cycles result in the contact angles converging to a value between that of the initial films. Once again, convergence of the contact angle values indicates the presence of a mixture of stilbene isomers on the substrate. Irradiation beyond three cycles did not result in any further significant change in the surface wettability.

Presence of the stilbene moiety on the substrate was confirmed after each irradiation cycle by grazing incidence IR analysis. Grazing IR spectra for the multilayered film and the first two irradiation steps are shown in Figure 5.2. A C=C stretch is observed at 1679 cm⁻¹ in all spectra. This signal and the other major IR absorbances are also observed in a sample of the solid compound examined by ATR, Figure 5.3. The signal at 1687 cm⁻¹ seen in the spectra for the multilayered films is due to the carboxylate of the monolayer complexed to the metal ion, and is also observed in the grazing IR of the deprotected SAM at 1691 cm⁻¹, shown in Figure 5.4. Further irradiation steps resulted in similar IR profiles. IR analysis was not able to differentiate
between the *cis*- and *trans*- isomers as the spectra of the multilayered films are indistinguishable.

![Absorbance spectra](image)

**Figure 5.1:** Absorbance spectra (normalized) of the *cis*- and *trans*- stilbene isomers in EtOH.

<table>
<thead>
<tr>
<th>Irradiation Cycle</th>
<th>Contact Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Irradiation</td>
<td>64° ± 2.0°</td>
</tr>
<tr>
<td>Cycle 1:</td>
<td></td>
</tr>
<tr>
<td>Irradiate at 254 nm</td>
<td>44° ± 2.0°</td>
</tr>
<tr>
<td>Irradiate at 350 nm</td>
<td>63° ± 2.0°</td>
</tr>
<tr>
<td>Cycle 2:</td>
<td></td>
</tr>
<tr>
<td>Irradiate at 254 nm</td>
<td>47° ± 2.0°</td>
</tr>
<tr>
<td>Irradiate at 350 nm</td>
<td>61° ± 2.0°</td>
</tr>
<tr>
<td>Cycle 3:</td>
<td></td>
</tr>
<tr>
<td>Irradiate at 254 nm</td>
<td>51° ± 2.0°</td>
</tr>
<tr>
<td>Irradiate at 350 nm</td>
<td>58° ± 3.0°</td>
</tr>
</tbody>
</table>

**Table 5.2:** Contact angle measurements for films prepared from photolabile monolayers.
Figure 5.2: Grazing IR of the multilayered film before and after irradiation.

Figure 5.3: IR of solid cis-stilbene dicarboxylic acid.
The major difference between films fabricated from mixed monolayers and those prepared from photolabile monolayers is that more irradiation is needed to cause converging contact angles in systems prepared by the latter method. Using a photolabile group to create space in the film for isomerization appears to be a superior approach to using a mixed monolayer based on the number of switchable cycles achieved. Any approach that favors isomerization at the expense of other photochemical deactivation routes (e.g., photodimerization) will result in more efficient isomerization over more irradiation cycles.
CONCLUSIONS

Reversible and irreversible photoinduced changes in surface wettability were observed in non-covalently assembled multilayered films. The multilayered films studied were fabricated from a SAM consisting of 4-(10-mercapto-decyl)-pyridine-2,6-dicarboxylic acid on gold, Cu(II) ions complexed to the pyridine head group of the SAM, and either cis- or trans- stilbene-4,4’-dicarboxylic acid complexed to the Cu(II) ions. Irradiation of these films at wavelengths corresponding to the absorption band of the respective stilbene isomer resulted in an irreversible chemical change as indicated by surface contact angle and grazing incidence IR measurements. However, no evidence for cis/trans- photo-isomerization was observed.

Thin films consisting of an underlying SAM, an intermediate layer consisting of Cu(II) ions and either cis- or trans-stilbene-4,4’dicarboxylic acid as the capping ligand, were fabricated with a mixed SAM that contained both 4-(10-mercapto-decyl)-pyridine-2,6-dicarboxylic acid and 4-tert butylbenzenethiol. Irradiation of these films at wavelengths corresponding to stilbene isomer absorption bands resulted in reversible cis- to trans- and trans- to cis- photo-isomerization, and reversible switching of the surface wettability between a hydrophobic (cis-stilbene) and a hydrophilic (trans-stilbene) state. The difference in observed behavior between these films and those described above is attributed to the greater surface spacing afforded by the mixed monolayer, which allows greater conformational flexibility and lowers the steric barriers to isomerization.

Surface separation was also achieved through the use of a monolayer with a photolabile group.
A SAM of 2-nitrobenxyl 11-mercaptoundecanoate was deprotected with UV light. Following capping with metal and stilbene-4,4’-dicarboxylic acid, isomerization was achieved for three complete cycles. The use of the photolabile group for monolayer preparation provides a uniform surface for multi-layer fabrication and, as evidenced by contact angle measurements, provides a superior template (compared to mixed monolayers) for the fabrication of a non-covalent film capable of photo-switchable wettability changes.
5.5. *Increased Wettability Changes.*

The change in contact angles achieved in these studies was approximately $20^\circ$. Although this is an improvement over systems that have reported wettability changes on the order of $9^\circ$ \cite{3}, it does not represent a change between ideal hydrophobic and hydrophilic monolayers. For example, CF$_3$ terminated SAMs exhibit contact angles of approximately $120^\circ$, and COOH terminated SAMs give contact angles on the order of $20^\circ$. A system that exhibits a change of $\sim 100^\circ$ in contact angle would provide a superior gate in a fluidic system.

Future work includes developing systems that undergo larger reversible wettability changes. This can be accomplished by fabricating photoisomerizable units with functional groups that have a more significant influence on the surface wettability depending on their orientation. For example, in this system a stilbene substituted with a carboxylic acid is used. However, in the *trans*- state the contact angle of $\sim 45^\circ$ does not indicate that the carboxylic acid group is fully exposed to the surface, as it is likely not in a vertical orientation. Similarly, in the *cis*- state the contact angle of $\sim 70^\circ$ is not what is expected of a purely hydrophobic alkyl group exposed to the surface (CH$_3$ terminated films result in a contact angle of $\sim 110^\circ$).

In order to effect a larger change in wettability, stilbene moieties with different functional groups on one of the aromatic rings could be used. For example, a CF$_3$ group in the ortho or meta position to the C=C on the ring may decrease the wettability of the film (larger contact angle) when the stilbene isomer is in the *cis*- orientation and provide a greater difference in
wettability when the isomerization process occurs. Examination of stilbene isomers with CF₃ and COOH functional groups in different positions of the ring system would provide more significant wettability differences for the multilayered film assembly.


Utilizing SAMs with bulky protecting groups to create surface separation has been reported previously and was used in these switchable wettability studies. However, the degree of separation and the structure of the monolayer following irradiation were not probed in detail; evidence for surface separation of binding sites was inferred by the changes in wettability following isomerization. A detailed study of the packing arrangement of the irradiated film following removal of the protecting group to determine how this method achieves reactive site separation is of interest for future studies. An understanding of the degree of surface separation can then be used to design future multilayered systems that undergo wettability changes since the increased spacing decreases the steric hindrance to isomerization.

In order to study the nature of the packing in photolabile monolayers, direct comparisons will be made between irradiated SAMs that have a carboxylic acid terminal functionality and those deposited from mercaptoundecanoic acid. These SAMs are chemically equivalent, the only difference being that the irradiated films likely have fewer molecules attached as they were formed with bulky terminal groups. Contact angle and CV measurements of these films are identical; however these types of measurements only provide a survey of the entire surface and do not give significant information about the packing of the individual chains, other than to show that they are well oriented. Thickness measurements of each of the films by ellipsometric or
capacitive techniques would determine if the monolayers have the same tilt or cant angle on the substrate. For films fabricated using bulky protecting groups, if the chains are separated by a larger distance prior to irradiation, they may tilt to a higher degree to establish maximum interchain interactions after irradiation. This would result in a decrease in the film thickness compared to a SAM prepared from mercaptoundecanoic acid. Determination of the difference in film thickness between these SAMs would allow for the difference in tilt angle to be calculated, and therefore demonstrate that an increase in separation between chains is achieved by using photolabile head groups.

Another relevant study is to examine the contact angle hysteresis between these two types of SAMs (COOH terminated groups formed using photolabile monolayers and those formed using mercaptoundecanoic acid). Contact angle hysteresis (described in Chapter 2) is the difference between advancing and receding contact angles, and the larger the hysteresis the more inhomogeneous the surface. The same contact angle hysteresis for these films would indicate a similar surface arrangement, while a larger hysteresis value for the films prepared using photolabile groups would indicate that the surface is less homogeneous and support the hypothesis that removal of the protecting group increases spacing between reactive sites. A less tightly packed monolayer would be expected to have a larger hysteresis as the terminal functionalities would be further apart reducing uniformity. The extreme case can also be tested by using mixed films (mercaptopundecanoic acid with short chain t-butyl thiol spacers) which will provide a less homogeneous surface and an even larger hysteresis.
EXPERIMENTAL DETAILS

Materials and Methods.

*Cis*-stilbene-4,4’-dicarboxylic acid (95 %) from Lancaster Synthesis Inc. (Windham, NH). *Trans*-stilbene-4,4’-dicarboxylic acid from Frinton Laboratories, (Vineland, NJ). Suppliers for other chemicals and details for methods of instrumental analysis are provided in Chapter 4.

Preparation of Mixed Monolayers and Multilayered Films.

Monolayers were prepared by immersion of the gold slides in a 1-2 mM solution of 4-(10-mercapto-decyloxy)-pyridine-2,6-dicarboxylic acid or a 5 mM solution of 2-nitrobenzyl 11-mercaptopoundecanoate in ethanol for 18 hours. Mixed monolayers were prepared by immersing the clean gold slides in a solution containing a mixture of 0.5 mM of 4-(10-Mercapto-decyloxy)-pyridine-2,6-dicarboxylic acid and 0.5 mM of 4-tert butylbenzenethiol in ethanol (for mixed monolayers the total concentration of thiol was 1 mM). Copper (II) bromide was used as the source of Cu (II) ions. SAMs were immersed in a solution of 2 mM copper bromide in ethanol for 2 hours. Capping of the copper ions with the *cis-* and *trans-* stilbene-4,4-dicarboxylic acid was carried out by immersing the copper capped gold slides in 5 mM solutions in ethanol. In the mixed monolayer systems following deposition of Cu (II), the gold slides were immersed in a solution of 2 M HNO₃ for 30 minutes and then rinsed with water followed by ethanol to remove any copper ions that may have physisorbed on the CH₃ head group. Control experiments showed no monolayer degradation following exposure to 2 M HNO₃. After each layer deposition, and prior to any characterization, the films were removed from solution, rinsed with ethanol, and dried with nitrogen. New films were prepared for each characterization experiment.
Preparation of Photolabile Monolayers and Multilayered films.

Monolayers of the photolabile compound (2-nitrobenzyl-11-mercaptopoundecanoate) were prepared and irradiated as described in Chapter 4. Multilayered films were prepared in a similar method to that described above. Irradiated SAMs were immersed in a solution of 2 mM copper bromide in ethanol for 2 hours. Capping of the copper ions with the cis- and trans- stilbene-4,4-dicarboxylic acid was carried out by immersing the copper capped gold slides in 5 mM solutions in ethanol. Following each deposition step the slides were removed removed from solution, rinsed with ethanol, and dried with nitrogen.

Irradiation.

Slides were irradiated using a Rayonet reactor equipped with mercury arc lamps with maximum distributions of light centered at 254 and 350 nm. Exposure to 254 nm light was performed with the slides in ethanol with no filter; for irradiation at 350 nm a Pyrex filter was used which cuts off light below 305 nm.
**Synthetic Details**

4-(10-mercapto-decyloxy)-pyridine-2,6-dicarboxylic acid.

This compound was synthesized by Dr. Ernesto Soto and the details are described in his doctoral dissertation and in previous publications.\textsuperscript{117-118}

2-nitrobenzyl 11-mercaptoundecanoate.

The synthesis of the photolabile compound is described in detail in Chapter 4.
6: Multi-Analyte Sensor Device Platform
INTRODUCTION

This chapter describes the individual components of a multi-analyte sensor device platform (electrode array, microfluidic system, sensor analogues) towards the application of building a portable blood sensing device. As a proof-of-principle for the device design crown ether analogues were synthesized and tested in response to alkali metal ions (K$^+$, Na$^+$, Li$^+$). As described previously, monitoring of electrolyte levels in biological fluids is important in identifying illness (K$^+$ and Na$^+$ levels) and also when certain therapeutics are used (Li$^+$ therapy). The device design presented demonstrates an electrochemical response to metal ions in solution, indicating it can be used as a platform for the attachment of other surface based sensors.

The same format was also tested for glucose sensing by incorporating the enzyme glucose oxidase onto the surface based electrode array. In the presence of glucose a portion of the enzyme reacts and is reduced which can be monitored electrochemically in the presence of a charge transfer mediator. As previously described, careful monitoring blood glucose levels is vital to diabetic patients.

Development of a sensing platform that functions with different types of surface based sensors (different crown ethers, surface bound enzymes) is a step towards fabricating a multi-analyte device. Demonstrating that the sensing platform is reusable (i.e. shows reproducible response to multiple samples) will lead to the development of a continuous monitoring device that can be used observe and examine physiological indicators of workers or soldiers in the field and alert medical personnel to a health problem prior to it becoming life threatening.
Sensors have applications that range from electronics, to manufacturing, to detection of toxic materials, to healthcare. A sensor is a device which has a response to an external stimulus. The response is transduced into a measureable signal that can be detected. Sensor responses include electrical (e.g. potential change), optical (e.g. color change), and thermal (e.g. temperature change) signals that are used to determine analyte levels. Detecting components (electrolytes, glucose) of physiological fluids (blood, urine) is important for physicians to evaluate patients and treat disease. Portable devices that rapidly assess physiological samples eliminate the requirement to collect and send blood or urine to a laboratory for analysis. Advantages of portable sensing systems in the healthcare field include reducing patient treatment times, eliminating laboratory expenses, and the ability to monitor analyte levels in remote (away from a hospital) locations.

A commercially available device, the i-STAT manufactured by Abbott Point of Care, is a handheld blood analyzer that performs a panel of tests for physiological levels of electrolytes, blood gasses, glucose, cardiac markers, and other blood chemistries. The i-STAT uses a series of self-contained cartridges to test samples; the individual cartridge is selected based on the type of blood chemistry analyzed. Different cartridges are available, some test for only a single analyte such as creatinine (an indicator of renal function), while others test for several levels such as electrolytes, (Na⁺, K⁺, Cl⁻) glucose, pH, and hemoglobin (a protein that transports oxygen from the lungs to the rest of the body. A drop of blood is applied to the cartridge which is inserted into a handheld analyzer and the test is completed in several minutes.

Although the i-STAT is a portable detection system for a range of blood chemistries, it has
several significant disadvantages. The first is cost. Individual cartridges can only be used for a single test and vary in price from about $10 to $40;\textsuperscript{200} this expense per sample can be prohibitive for a large volume of measurements. Also, unexpected readings cannot be retested with the same cartridge; a new cartridge (with the additional cost), or laboratory analysis to confirm the initial measurement is required. The response time of several minutes is satisfactory for single analyte measurements (such as the level of glucose in a blood sample), but the system cannot be used for real-time detection that is necessary for a continuous monitoring device. Applications of continuous monitoring include implantable devices that monitor soldiers in the field and alert others in a remote location of changes in their blood chemistry (such as electrolyte levels) that require immediate attention.

The goal of the work presented in this chapter is to develop a compact, portable sensing platform capable of multi-analyte detection, using minimal sample volumes, for biological sensing applications. Specific aims include: the design and fabrication of a device (containing a surface based electrode array, a microfluidic sample delivery system, and an electrical interface to a potentiostat/frequency response analyzer for detection), synthesis of crown ether analogues that bind alkali metal ions and can be attached to surface based electrodes, testing of the ion binding affinity of sensor compounds in a multiplexed format, and detection of analyte in the device platform.

Surface based sensors that are chemically bound have advantages for incorporation into devices, particularly in reusability because of the permanent attachment to the substrate.\textsuperscript{201-203} Examples of surface sensors include cyclic and bicyclic crown ethers and porphyrins, which provide
suitable sites for metal ion interactions.\textsuperscript{62,204-205} A diagram of a surface based sensor of this type is shown in Figure 6.1. Upon binding ions, the surface properties (e.g. resistivity) of the sensor molecule change and this response can be measured.

![Surface based sensor diagram](image)

**Figure 6.1:** A surface based sensor showing an ion binding site and spacing linker for surface attachment.

Surface based sensor arrays have been used for detecting metal ions,\textsuperscript{206-207} enzymes,\textsuperscript{208} bacteria,\textsuperscript{209} trace gasses,\textsuperscript{201,210} and organics.\textsuperscript{60,203,211-215} Techniques that have been used to prepare these types of arrays that include; photoligthography,\textsuperscript{216} self-assembly,\textsuperscript{206-207,209,211} the use of polymer matrixes,\textsuperscript{212,214} the controlled growth of carbon nanotubes,\textsuperscript{210} the controlled growth of nanowires,\textsuperscript{217} and microfabrication.\textsuperscript{60,201,208-209,215} Often, more than one of these techniques are used to generate the array, resulting in a complicated, time consuming fabrication process; simplifying the sensor fabrication process using spontaneous molecular interactions will reduce cost for a medical device.
Self-assembled monolayers of alkanethiols on gold have been studied for biosensing.\textsuperscript{3} As previously discussed, gold is compatible with biological systems and electrically conductive. Interaction between terminal functional groups of the adsorbate with analyte species have led to the use of SAMs in selective molecular recognition applications including chemical and biological sensing.\textsuperscript{55,62} Analyte molecules bind to the monolayer and the extent of binding is transduced into a detectable signal through bulk modulation of properties including fluorescence, surface plasmon resonance, or electrochemistry.\textsuperscript{55,62} Direct transduction of a molecular binding event into an electrical signal makes electrochemical detection appealing as it can be integrated into portable electronic devices. Monolayers that incorporate metal ion binding sites have been shown to detect ions with a response that can be measured electrochemically.\textsuperscript{100-101}

Host-guest interactions are a type of sensing method. Typically, the host molecule contains some kind of central hole or some other type of cavity.\textsuperscript{198} To be an effective sensor, the receptor site is selective in its interaction with the analyte in the presence of other species. Also, the receptor is attached to a signaling unit that responds to the interaction with the analyte in one of the methods described above.

Crown ethers have been studied for their ability to interact with alkali metal ions in a host-guest fashion.\textsuperscript{218} The ion-dipole interaction between the lone pair electrons of the oxygen atoms and the positive charge of the cation, make crown ethers candidates for ion detection. Binding constants of crown ethers and alkali metal ions in aqueous media are on the order of $10^1 - 10^2 \text{ M}^{-1}$.\textsuperscript{198} A molecular modeling simulation of the interaction between 18-crown-6 and K\textsuperscript{+} in water is shown in Figure 6.2. If the crown ether is anchored to a surface, in the case of a self-
assembled monolayer, the interaction of metal ions with the crown moiety can be monitored electrochemically through the substrate.

![Figure 6.2: Molecular Modeling Simulation of Host-Guest Interaction of K$^+$ and 18-Crown-6 (Grey = C, Red = O, Purple = K$^+$)](image)

Electrochemical impedance spectroscopy (EIS), introduced in Chapter 2, has been used to examine properties of self-assembled monolayers including surface coverage, monolayer composition, and the ability of surface sensor molecules to selectively bind ions.\textsuperscript{95-98,100-101} Impedimetric sensing functions by detecting changes in the insulating behavior of a system.\textsuperscript{100-101} Lennox and co-workers demonstrated that impedance measurements of SAMs modeled to simple electrical circuits allow values for the resistive and capacitive components of the system to be extracted. Reinhoudt and co-workers have shown that the complexation of metal ions to self-assembled monolayers of crown ethers causes an increase in the monolayer capacitance, and also an increase of the charge transfer resistance in the presence of a redox active species, which can be monitored by impedance spectroscopy.\textsuperscript{62,100-101,204} This form of sensing can be used to directly detect redox inactive species without labeling.
A microfluidic testing format combines the advantages of electrochemical detection using microelectrodes with small sample volumes. The dimensions of microelectrodes have been shown to reduce response time, increase sensitivity and effect efficient mass transport. Microfluidic sample delivery allows for high-throughput analysis with minimum sample (µL) required and waste produced. Microfluidics also allows for parallel detection and/or sample pre-processing which is important for "lab on a chip" applications. A microfluidic SAM based sensor platform combines reduced costs with increased speed, sensitivity, and portability.
RESULTS AND DISCUSSION

6.1. Device Design and Fabrication.

The microfluidic device design includes several components. Three surface electrodes (working, counter, and reference) are interfaced to a potentiostat. A poly-dimethyl siloxane (PDMS) microfluidic channel delivers the analyte solution to the active electrode area of the device, and is fabricated by casting the polymer over a mold. Sample delivery tuning is inserted into the PDMS and a fixture secures the entire assembly.

6.2. Initial Prototype.

The initial device design included the components described above; a diagram and photograph of the prototype are shown in Figure 6.3. A 75 mm × 25 mm gold coated glass slide was used and three independent electrodes were created by etching the surrounding metal layer (aqua regia removed the gold and a commercial chromium etchant removed the adhesion layer). A PDMS channel was affixed to the top of the electrode array and clamped in place using a custom made acrylic fixture. Although this design contained the necessary components two critical problems were realized. First, a gold reference electrode is not sufficient as the potential of the system drifted during the measurements (see section 6.5). Second, the large size of the exposed electrode area compared to the small volume of electrolyte resulted in the gold layer detaching from the substrate during the measurements, as the current flow in the system was limited by the size of the counter electrode.
6.3. Device Miniturization.

To address the problems associated with the initial prototype the design of the system was modified. A microelectrode array was used to correct the size ratio between the electrodes and the volume of analyte solutions. Microelectrodes also keep the overall device platform small which is important for the portability of the system as well as maintaining minimal sample volumes. Dimensions of the microfluidic network and clamping fixture were scaled appropriately and are described in the experimental details at the end of the chapter.

6.4. Microelectrode Arrays.

A commercially available Electrochemical Cell-on-a-Chip (ECC) was used as the surface based sensing platform for analyte detection (details provided at the end of the chapter). The ECC consists of two active areas that each contain an interdigitated working electrode with
neighboring counter and reference electrodes. A diagram of the ECC used in this study is shown in Figure 6.4; total dimensions of the array are $20 \times 10$ mm. The inset in the figure shows an enlargement of the active area ($3 \times 3$ mm) of the array which contains the three electrodes.

![Figure 6.4: Diagram of Electrochemical Cell-on-a-Chip (ECC) (from ABTECH company website).](image)

This electrode design offers two critical components. The first is an independently addressable reference electrode that is in close proximity to the working electrode and can be electroplated with silver using an electroplating solution as described in the next section. The close proximity of the electrodes reduces resistance; the resistance between the two electrodes should be minimal to maintain a constant potential. The second advantage of the ECC is the large area counter
electrode compared to the working electrode. Typically, counter electrodes are 100 to 1000 times larger than working electrodes so that sufficient current can be transferred into the electrolyte while avoiding high cell voltages.

6.5. Electroplating of Reference.

Metallic silver was plated onto the reference electrode portion of the ECC to generate a stable reference for surface based electrochemical measurements. The electrode was placed in a silver plating solution and a constant current was applied to the reference portion of the ECC. During the plating process silver ions are reduced to silver metal and deposited on the electrode. Successful electroplating was confirmed by measuring the potential over time vs. a standard reference electrode (Ag/AgCl). These measurements demonstrate the stability of the electrode and a comparison between bare gold and gold plated with silver is presented in Figure 6.5. Additionally the silverized ECC reference demonstrates similar stability vs. a 1 cm$^2$ gold substrate plated with silver using the same process, as also shown in Figure 6.5. It is noted that plated silver surfaces are comparable to that of a silver wire in terms of stability vs. a standard reference electrode.
6.6. Assembled Device.

Photographs of the assembled device platform are shown in Figures 6.6 and 6.7. The microelectrode array is set in a custom made acrylic fixture as shown in Figure 6.6 (left); the two independently addressable active electrode regions are seen in the image. The image in Figure 6.6 (right) shown a PDMS channel placed on top of the electrode array. The top portion of the fixture (not shown) has a recess for the PDMS channel and is designed to center the channel over the active electrode array and affix the system to avoid electrolyte leakage. The complete assembled device with an electrical interface is shown in Figure 6.7; the blue clip makes an electrical connection with the contact pads of the electrode array and the black connector interfaces to the instrument. Approximate dimensions of the fixture are 3 cm × 3 cm. It is noted
that the total size of the electrical interface is larger than the sensing array and further miniaturization of the instrument connection will reduce the size of the platform.

Figure 6.6: Electrode array set in fixture (left) and electrode array covered by PMDS channel (right), tubing is inserted in the PDMS for sample delivery.

Figure 6.7: Assembled device, showing the electrode array in the clamping fixture and the electrical interface.
6.7. Synthesis of Sensor Compounds.

Three target compounds were identified as sensors for incorporation into the microfluidic device. The compounds consist of different sized crown ethers with a short chain thiol linker for surface attachment and are shown in Figure 6.8. Short linking chains provide a larger response upon ion binding because the overall impedance of the chain is reduced compared to longer linking groups typically used to form alkanethiol self-assembled monolayers.\textsuperscript{101} However, interactions between adjacent alkyl chains stabilize and orient monolayers on a surface,\textsuperscript{4} and using shorter chains may reduce the degree of order achievable for these films. Different sized crown ethers were chosen to increase the selectivity towards metal ions (specifically Li\textsuperscript{+}, Na\textsuperscript{+}, K\textsuperscript{+}) upon incorporation into a device. By analyzing the response of each of these sensor compounds to the same analyte, the selectivity is increased compared to using a single sensor.

Figure 6.8: Chemical structures of crown ether targets.
A synthetic approach to the three target compounds was developed from a modified literature procedure, and is shown in Scheme 6.1. The reaction involves treating a monoazacrown with γ-thiobutyrolactone in the presence of catalytic amounts of camphorsulfonic acid. An amide bond is formed and the lactone ring opens generating a free thiol group that can be used to link the compound to a gold surface. This one step reaction can be completed in 1 day, and is followed by purification. The same approach was used to prepare the three target compounds in amounts greater than 500 mg and the synthetic procedure is described in detail at the end of the chapter (spectroscopic characterization is provided in Appendix A).

**Scheme 6.1: Synthetic approach to the target compounds.**

SAMs were prepared using each of the crown ether compounds and were characterized by contact angle and grazing incidence IR measurements. Contact angles of 55.8° ± 3°, 56.8° ± 4°, and 56.8° ± 4° were determined for SAMs of the aza-12-crown-4, aza-15-crown-5, and aza-18-crown-6 short chain thiols, respectively. Similar contact angles are expected as each of the compounds has the same ether terminal moiety, and the measured angles are in agreement with reported values for films of similar structure.100-101

Grazing IR spectra for each of the crown ether SAMs are shown in Figures 6.9, 6.10, and 6.11. The three films show similar IR absorptions due to the similar chemical structure of the three compounds. Common absorptions include: ~ 1135 cm⁻¹ (C–O), ~ 1460 cm⁻¹ (C–N), ~ 1640 cm⁻¹ (C=O amide), ~ 2857 cm⁻¹ (C–H chain and C–H crown), ~ 2900 cm⁻¹ (C–H crown), and ~ 2928 cm⁻¹ (C–H chain). These absorptions are consistent with a SAM of each of the crown ether compounds forming on the substrate. The observation of the C–H stretches of the chain at ~ 2928 cm⁻¹ and ~ 2857 cm⁻¹ for all films are indicative of a somewhat disorganized, liquid-like arrangement of the molecules on the surface, expected due to the short tether chain length and bulky head group, and is similar to reports that discuss SAMs containing crown ether moieties.100-101
Figure 6.9: Grazing IR of a SAM of aza-12-crown-4 thiol.

Figure 6.10: Grazing IR of a SAM of aza-18-crown-6 thiol.
6.9. **Ion Titrations.**

Ion titrations were performed by adding aliquots of ion (Li\(^+\), Na\(^+\), and K\(^+\)) to a supporting electrolyte solution and monitoring changes in charge transfer resistance of the film by impedance spectroscopy. These measurements were performed on planar 1cm\(^2\) gold substrates. A supporting electrolyte of TRIS buffer (0.1 M) adjusted to physiological pH (7.4) along with a redox active species, hexamineruthenium chloride, was used. A positively charged redox probe is employed for these measurements because binding a positive ion to the film results in an electrostatic repulsion between the interface of the film and the redox probe in solution. The electrostatic interaction is evidenced as an increase in the charge transfer resistance of the film and is monitored by impedance measurements.
Results for ion titrations using these methods are presented in Figures 6.12 and 6.13, which plot changes in charge transfer resistance vs. ion concentration. Figure 6.12 shows the response of a SAM of aza-18-crown-6 thiol to alkali metal ions. The largest change in charge transfer resistance (~ 800 Ω at 25 mM) is observed for K\(^+\) ions and the smallest (~ 30 Ω for 25 mM) is seen for Li\(^+\) ions. Response of each of the different sized crowns to K\(^+\) ions is shown in Figure 6.13. Monolayers of aza-18-crown-6 thiol show the largest change (~ 450 Ω at 25 mM) in charge transfer resistance and those of 12-crown-4 thiol demonstrate the smallest (~ 0 Ω at 25 mM) change. These results are consistent with size-fit interactions of the alkali metal ions and crown ether analogues. It is noted that the initial charge transfer resistance values vary for these films between ~ 100 and ~ 300 Ω, and as a consequence the results are plotted as changes in the charge transfer resistance so that different systems can be compared. It is also noted that these values are obtained from modeling the experimental data to an ideal equivalent circuit, as described in detail in Chapter 2, which results a degree of error for the values obtained and explains why some of the films examined (for example 12-crown-4 thiol with potassium) show a small negative change in charge transfer resistance from one measurement to the next.
Figure 6.12: Impedametric ion titrations of aza-18-crown-6 thiol.

Figure 6.13: Impedametric ion titrations of crown ether SAMs with K⁺.
Initial ion titration results were encouraging; differences in responses were observed between the crown ether modified electrodes. To demonstrate multi-analyte sensing, a multiplexed experiment was designed to allow measurement of the same analyte solution with all three crown ether constructs.

6.10. *Multiplexed Ion Titrations.*

A manual multiplexer was fabricated that consisted of three working electrode channels with a single connection to the working electrode lead of the potentiostat. Each channel is independently selectable with a switch, and the multiplexer allows for three working electrodes (the three crown ether SAMs) to be integrated in one electrochemical cell with common reference and counter electrodes. Measurements are performed in sequence by adjusting the ion concentration of the electrolyte, selecting one of the working electrodes for measurement, and then repeating the measurement with the other two working electrodes. This arrangement is analogous to a multi-analyte sensor device in that the same analyte solution is analyzed by a set of modified electrodes with different chemistries.

Multiplexed titrations of the crown ether analogues with alkali metal ions are presented in Figures 6.14 (K⁺), 6.15 (Na⁺), and 6.16 (Li⁺). The plots show the change in charge transfer resistance vs. ion concentration and each graph contains data from three separate sets of SAM modified electrodes for a total of 9 measurements (3 with each crown) at each concentration. Although there is a significant difference between the values measured for each crown with each metal ion (for example the change in charge transfer resistance for aza-18-crown-6 thiol varies
by ~ 150 Ω for each ion at the maximum concentration measured) several trends are observed.

For potassium and sodium the largest crown (18-crown-6) shows the largest increase in charge transfer resistance (between 250 and 500 Ω at the maximum concentration measured) and the smallest crown (12-crown-4) shows the smallest increase (between 15 and 50 Ω at the maximum concentration measured). For lithium, both the 18-crown-6 and 15-crown-5 modified electrodes show similar responses (increases of 130 to 230 Ω at the maximum concentration), while the 12-crown-4 surfaces exhibited changes between 70 and 110 Ω at 25 mM. This result is significant because the other ions (Na⁺ and K⁺) produced a response of 12-crown-4 substrates that was below 50 Ω (most measurements gave changes of ~ 20 Ω for the maximum concentration).

![Figure 6.14: Multiplexed titrations of crown ethers with K⁺.](image-url)
Figure 6.15: Multiplexed titrations of crown ethers with Na\(^+\).

Figure 6.16: Multiplexed titrations of crown ethers with Li\(^+\).
Multiplexing the ion titrations in this manner demonstrates how multiple sensors are used to take measurements of the same analyte solution in sequence. This method of analyte detection improves the selectivity over one sensing unit alone. For example, if sensors A and B both respond to analyte C, but only sensor A responds to analyte D, a device that uses both sensors A and B will detect both analytes C and D. The response of neither indicates no analyte C or D present, the response of A only indicates analyte D, and the response of both A and B indicates analyte C.


A microfluidic device was assembled with an electrode array derivatized with aza-18-crown-6 thiol. Measurements were taken by injecting alternating solutions of supporting electrolyte (TRIS buffer and hexaamineruthenium chloride) with 0 mM K\(^+\) and supporting electrolyte with 5 mM K\(^+\) into the device and monitoring the impedance after each injection. The normal physiological concentration of potassium is 3.5 to 5.0 mM\(^{63}\) and a useful biological detection system is responsive in this range. A plot of the imaginary vs. the real components of the impedance for 9 alternating cycles is shown in Figure 6.17. The initial charge transfer resistance is 6.8 kΩ and an increase to 9.9 kΩ is observed upon addition of 5 mM K\(^+\), which is consistent with the metal ion binding to the film and causing an electrostatic repulsion between the surface interface and redox species in solution. It is noted that at metal ion concentrations of 5 mM the diffusion limited part of the impedance spectra (Warburg element seen for 0 mM ion concentration) could not be recorded due to the high charge transfer resistance, which has been reported for similar systems.\(^{100}\) In this case the low frequency points were removed from the plot for clarity as the impedance at these frequencies was similar to those depicting the charge
transfer resistance of the film (in effect the overall impedance at these frequencies is the same as that of the lowest frequency point shown in Figure 6.17).

After flushing the device with supporting electrolyte with no ion, the charge transfer resistance decreases to 7.3 kΩ, within ~ 500 Ω of the initial value. The irreversible increase in charge transfer resistance observed for the first cycle is either due to potassium ions that remain bound to the film or a reorganization of the film as a result of the AC perturbation during the measurement. The second explanation is more likely because subsequent cycles demonstrate charge transfer resistance values that return to a range within 50 Ω of 7.3 kΩ (the value measured after the first cycle).

Figure 6.17: Impedance measurements of ion binding in a microfluidic device at alternating concentrations of 0 and 5 mM K⁺, series 1-9 indicate the order of the measurement.
The other electrode of the ECC showed similar reversible behavior. An initial charge transfer resistance value of 8.3 kΩ was measured and upon addition of 5 mM K\(^+\) the value increased to 14.6 kΩ. A similar irreversible increase in the charge transfer resistance was noted after the first cycle, but further cycles saw a reversion to within ~ 50 Ω of that value, analogous to electrode 1. Although the trend for both electrodes is the same, the initial resistance values vary by about 1.5 kΩ, and the change in charge transfer resistances at 5 mM K\(^+\) ion concentration is different by 3.3 kΩ.

As observed during the multiplexed titrations, films with higher initial resistances show a greater change in charge transfer resistance when exposed to ion, which likely has to do with the organization of the film. Higher initial values indicate a well ordered monolayer (comparatively) with fewer defects. Defect sites in the film allow penetration of the redox species regardless of the degree of ion binding and as an effect lead to comparatively lower changes in resistance values. For this system the electrode with the lower initial charge transfer resistance (6.8 kΩ) saw an increase of 3.1 kΩ at 5 mM K\(^+\), while the electrode with the higher initial resistance (8.3 kΩ) produced a change of 6.3 kΩ at 5 mM K\(^+\).

The reversibility of the system seen in both measurements in the microfluidic device and in those described above using 1 cm\(^2\) substrates and a standard electrochemical cell indicates the binding constant for the surface bond sensors and alkali metal ions is low. Electrodes were only rinsed with deionized water to remove bound ion and a high binding constant would prevent complete removal of the ion from the film. A low binding constant for surface bound crown ethers can be explained in that the hydrophobic boundary at the surface makes the diffusion of solvated alkali
metal ions into the film difficult, and does not result in a strong binding interaction that would be observed if both the crown ethers and ions were in solution. The low ion binding constant is an advantage in the design of a reversible sensing system, which necessitates that the analyte be capable of binding to the film for detection and also be easily removed from the film so that additional measurements can be performed on the same surface.


To further demonstrate the utility of the sensing platform, glucose oxidase was deposited on a gold substrate as a multilayered film and used for glucose detection in a similar method to that reported by Shervedani and Hatefi-Mehrjardi. Due to experimental limitations the measurements were performed in a standard electrochemical cell rather than in a microfluidic device format. However, these experiments were used as a proof-of-principle that the device platform can be used for glucose detection in addition to alkali metal ion detection.

In order to demonstrate that the surface based electrode configuration is suitable for the electrochemical detection of glucose, a series of measurements was performed prior to using the ECC. These experiments were undertaken to show that both a gold counter electrode can be used in place of platinum and that a plated silver reference can be used in the place of a standard Ag/AgCl reference electrode. Additionally, initial measurements were also used to analyze if the exposure of the surface based reference and counter electrodes to the glucose oxidase attachment procedure would affect the electrochemical measurements. These initial measurements were done using 1 cm² gold substrates that were cleaned and chemically modified with the same procedure used for functionalizing the ECC. Bare gold substrates, silverized gold substrates, and
silverized gold substrates exposed to glucose oxidase were also prepared. The measurements were first done using a gold substrate functionalized with glucose oxidase as the working electrode, a platinum wire counter electrode, and a Ag/AgCl reference electrode, in the absence and presence of glucose in the electrolyte solution. Then, the counter and reference electrodes were sequentially replaced with gold and silver substrates respectively. Finally, gold and silver substrates functionalized with glucose oxidase were used. The measurements demonstrated identical response of the system to glucose for both standard platinum counter and Ag/AgCl reference electrodes and gold and plated silver substrated exposed to glucose oxidase. This result indicates that measurements using the surface based ECC will be analogous to those using a standard three electrode cell.

The process for glucose detection by the functionalized substrate and the resulting electrochemical response is as follows. Surface bound glucose oxidase reacts with glucose in solution at the electrode surface which reduces FAD of the enzyme to FADH$_2$ which is immobilized in position in the enzyme and cannot migrate to the electrode surface to be oxidized. A charge transfer mediator (p-benzoquinone, PBQ) in solution transfers the charge from the immobilized glucose oxidase enzyme to the electrode surface to oxidize the FADH$_2$. This process can be monitored by impedance spectroscopy as a decrease in the charge transfer resistance of the redox probe (PBQ) upon exposure of the system to glucose. This method is in contrast to that used for alkali metal ions which were detected by an increase in charge transfer resistance as positively charged metal ions were bound to the film.

Measurements were performed by exposing the functionalized ECC to increasing glucose
concentrations from 0 to 50 mM in the presence of a PBQ redox probe and monitoring the impedance of the system. A plot of the overall impedance (imaginary vs. real components) is shown in Figure 6.18. A decrease in the overall impedance is observed as the charge transfer resistance decreases due to the PBQ mediating the oxidation of a portion of the substrate bound enzyme. It is noted that detection is reversible, exposure of the ECC to a solution with 0 mM glucose results in an increase of the overall impedance to the initial value.

Figure 6.18: Impedance measurements of a glucose oxidase modified electrode upon exposure to glucose solutions (0 to 50 mM)
CONCLUSIONS

A sensor device platform for multi-analyte detection has been developed. The device is composed of a surface based electrode array that is functionalized with sensing moieties, a microfluidic sample delivery system, and an electrical interface to measure analyte binding. Advantages of this design are that it is reusable, portable, and uses small sample volumes.

Three crown ether compounds were selected, synthesized, and tested for incorporation into a sensing system. Multiple sensing units in the same device increase the overall selectivity of the system, particularly important for detecting alkali metal ions which are difficult to discriminate by molecular recognition. Ion binding behavior of the crown ether SAMs was investigated by impedance spectroscopy. Impedance analysis demonstrated that the crown ethers respond differently to alkali metal ions due to size-fit complexation interactions, and the measurements were multiplexed to illustrate multi-analyte detection. Potassium ion binding was detected with aza-18-crown-6 thiol using the microfluidic device. The system demonstrated reversible detection of K\(^+\) ions in the physiological relevant range. Glucose oxidase was also incorporated into a surface based electrode system and tested to demonstrate reversible detection of glucose in the physiological range of 0 to 50 mM. Incorporation of more than one sensor into this platform will allow for monitoring of multiple physiological relevant species such as electrolytes, glucose, and blood urea nitrogen compounds in a single device.
**Future Work**


The crown ether surface based sensor compounds described in this chapter are different sized monoazacrowns connected to a four carbon thiol linker through an amide bond. These compounds were chosen because they offered a one-step synthetic route to three different compounds for incorporation into a multi-analyte device. However, the surface arrangement, and therefore ion binding affinity, of these structures is not known, and these compounds will be directly compared to those of similar structure using ion titrations analyzed by impedance spectroscopy to improve sensor selectivity.

Factors to be considered include chain length, coupling chemistry to the crown, and composition of the crown (oxygen/nitrogen atoms). Studies have examined the odd/even effect (number of alkyl groups in the chain) of SAMs and showed that different surface properties, such as wettability, result for SAMs from the same analogue with a different number of carbons in the linking chain (9 vs. 10 for example). This odd/even effect may also influence surface sensors, and a comparison between crown ethers bound to a substrate by both 3 and 4 carbon chains is of interest (short chains are necessary in order to observe changes in charge transfer resistance upon ion binding). Investigations of the coupling chemistry to azacrowns include comparing coupling through an amide bond (those described above) to coupling using an amine bond. The presence of the carbonyl group likely influences the surface packing as well as ion binding; however, whether the additional oxygen atom would enhance or diminish the analyte detection capability is unknown. Crown ethers containing nitrogen atoms are less selective for alkali metal ions than...
those containing only oxygen atoms;\textsuperscript{218} although it is more difficult to synthesize crown ether analogues that have no nitrogen atoms in the crown on which to attach a thiol tether.

Four 18-crown-6 derivatives are shown in Figure 6.19. Structure A has been synthesized and tested (described in Chapter 6) and uses an amide bond to attach the crown to the thiol tether. Structure B includes a carbon-nitrogen (amine) linking bond and can be synthesized from 1-aza-18-crown-6 as a starting material. Structure C has a linking ether chain and can be prepared from 2-(hydroxymethyl)-18-crown-6, which is commercially available. Structure D contains only carbon-carbon bonds in the linking portion and is more difficult to prepare because the crown ether ring must be formed during the synthesis. Structures A – D shown in Figure 6.19 represent 18-crown-6 derivatives that each have a 4 carbon chain (in the case of C, 3 carbons and 1 oxygen) linking the crown to the thiol, and a set of analogous compounds with a three atom linking chain can also be prepared. This total of 8 compounds would allow for direct comparison of surface ion binding behavior based on chain length, coupling chemistry, and crown composition.

Structures with other crown sizes (12-crown-4, 15-crown-5, 21-crown-7) are also envisioned. A binding study of all 32 proposed crown structures with alkali metal ions (Li\textsuperscript{+}, Na\textsuperscript{+}, K\textsuperscript{+}) would provide information that would assist in designing selective ion sensing devices. Comparisons between different structures can be made by estimating association constants from changes in charge transfer resistance values using the equation:\textsuperscript{101}

\[
Kc = \frac{R_{et}}{R_0} - 1
\]
where $K$ is the association constant of the metal ion, $c$ is the concentration of the metal ion, $R_{ct}$ is the charge transfer resistance in the presence of ion, and $R_0$ is the charge transfer resistance of the monolayer with no ions.

Figure 6.19: 18-Crown-6 derivatives.
Preliminary work has identified a synthetic scheme to the three carbon chain version of Structure B of Figure 6.19, and a portion of the synthesis has been completed. The synthetic route to the target compound proceeds in 4 steps and is shown in Scheme 6.2. The three carbon version was investigated because step 1 was reported previously; a 4 carbon version can be prepared using the same method. Steps 1 and 2 have been completed and the products have been purified and fully characterized. Steps 3 and 4 have been attempted; however a purification procedure for the products has not been determined.

Step 1 involves the synthesis of s-acetyl-3-mercapto propanol by combining allyl alcohol and thioacetic acid in the presence of an initiator, azoisobutyronitrile (AIBN). In step 2 the alcohol is oxidized an aldehyde, s-3-oxopropyl ethanethioate, using a Swern oxidation, named for the chemist who developed it, Daniel Swern. A Swern oxidation involves combining oxalylchloride and DMSO to generate an activated species (dimethylchlorosulfonium chloride) that oxidizes the alcohol when it is introduced. The activated species is only stable at low temperatures and necessitates cooling the reaction to –78 °C. A Swern oxidation is efficient at oxidizing alcohols to aldehydes without any further oxidation to carboxylic acids. Both the alcohol and aldehyde have been synthesized, purified, and characterized, and the procedures are described in detail at the end of this chapter (NMR data is provided in Appendix A).

Step 3 is reductive amination to couple the aldehyde to the monoazacrown. Several coupling methods were attempted, which included different reducing agents such as sodium cyanoborohydride and also H2 with a Pd catalyst, and were all unsuccessful. A procedure that
uses sodium triacetoxyborohydride in dichloroethane proved to be an effective method. Step 4 is a deprotection of the acetyl group using ammonium hydroxide, yielding the target compound.

**Scheme 6.2: Synthetic route to an 18-crown-6 derivative.**

**Step 1**

\[
\text{CH}_2\text{OH} + \text{CO}_2\text{SH} \xrightarrow{\text{AIBN, N}_2, \text{rt}} \]

**Step 2**

\[
\text{O} \xrightarrow{\text{Oxalylchloride, DMSO, -78 C}} \]

**Step 3**

\[
\text{O} \xrightarrow{\text{Sodium Triacetoxyborohydride, Dichloroethane, N}_2, \text{rt}} \]

**Step 4**

\[
\text{NH}_4\text{OH} \xrightarrow{\text{Ethanol}} \]

**Product**

\[
\text{HS} \]
Future work includes determining a solvent system to purify the product of step 3 by column chromatography. Preliminary reactions were carried out on a small scale (100 – 500 mg of the crown ether) due to the cost of the starting material. These reactions yielded very little crude product (less than 100 mg) for purification attempts by column. Alternatively, preparative HPLC or flash chromatography could be used. This synthetic scheme will be varied to prepare crown ether derivatives of different sizes, as well as different chain lengths, such as those described above.


Sensor devices that respond both electrochemically and optically to analyte binding offer advantages over those that only have one detection method. Devices with dual transduction mechanisms have reduced error, and provide for a means of self-calibration because the binding event causes more than one change in the system. Electrochemical methods of detection have been described in detail above. Optical detection can be accomplished by incorporating fluorophores into the ionophore structure and measuring changes in fluorescence emission behavior upon exposure to ion.

A target compound capable of dual transduction mechanisms that can be attached to a gold surface is shown in Figure 6.20. Ion binding results in changes in the capacitive and resistive properties of the film, similar to those described in the previous chapter. Optically, reversible ion binding results in ‘off-on’ fluorescence behavior. Photo-electron transfer from the lone pair of the nitrogen atom quenches the anthracene fluorescence when no ion is present, however when the lone pair is involved with ion binding, the fluorescence of the anthracene will be
observed. This behavior has been observed in similar crown ether/anthracene compounds in solution.\textsuperscript{223-224}

![Compound Image]

**Figure 6.20: Target compound for dual transduction.**

Preliminary work has identified a synthetic procedure to a portion of this compound and is presented in Scheme 6.3;\textsuperscript{225} the product has been prepared and characterized. Step 1 involves protecting one of the amine groups in the diazacrown; purification is required to separate the mono-protected and di-protected species (control of the reaction stoichiometry will limit the amount of disubstituted product formed). In step 2 the protected diazacrown is reacted with chloromethyl anthracene to generate a protected anthryl crown. Protecting the diazacrown prior to addition of the anthracene moiety is advantageous to avoid having to separate mono- and disubstituted anthryl compounds, which is difficult because of the instability of the anthryl crown on a silica column.\textsuperscript{223} Deprotection can be accomplished with trifluoroacetic acid in step 3 to regenerate a free amine group that is then available for further functionalization. The synthetic
procedure for this portion of the target compound is described in detail at the end of this chapter, and NMR data for each of the reaction steps is provided in Appendix A.

Scheme 6.3: Synthetic route to a portion of the target compound.

**Step 1**

\[
\begin{align*}
\text{NH} & \quad \text{HN} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O}
\end{align*}
\]

\[+\quad \text{Boc}_2\text{O} \rightarrow \text{Dioxane, rt}\]

**Step 2**

\[
\begin{align*}
\text{NH} & \quad \text{N-Boc} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O}
\end{align*}
\]

\[+\quad \text{Butyronitrile, reflux}\]

**Step 3**

\[
\begin{align*}
\text{N-Boc} & \quad \text{N-Boc} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O}
\end{align*}
\]

\[\rightarrow \text{TFA, rt}\]

**Product**

\[
\begin{align*}
\text{HN} & \quad \text{HN} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O}
\end{align*}
\]
Future work includes coupling a thiol chain to this compound for surface attachment. Two synthetic routes have been identified and are presented in Schemes 6.4 and 6.5. Synthetic route 1 (Scheme 6.4) involves forming an amide bond using thiobutyrolactone, analogous to the method for preparing the crown ether derivatives described previously. Route 1 is a one step method, and has been demonstrated to be feasible for preparing similar compounds (monoazacrown ethers). Synthetic route 2 (Scheme 6.5) uses a reductive amination to form an amine bond, in an identical method to that described in Section 6.13. Preparation of the target compound by both routes will allow for a comparison between amide (O=C-N) and amine (CH₂-N) coupling to the thiol tether.

**Scheme 6.4: Synthetic route 1 to target compound.**
Scheme 6.5: Synthetic route 2 to target compound.
6.15. Implementation of the Sensor Platform.

Implementing the sensor platform as a diagnostic device requires investigation of additional components that have not yet been explored. These include: a sampling head (for sample pretreatment and delivery), a new electrode array (with more active sensing areas), and a portable electrochemical detection system (for handheld analysis). The ideal system will allow the operator to place a drop of biological sample (such as blood or urine) into the device and get an accurate measurement of analyte levels (such as Na\(^+\), K\(^+\), NH\(_4\)\(^+\), or glucose) in a remote location (away from a hospital or laboratory).

The sampling head has two functions: pretreat a biological sample such as whole blood for analysis, and deliver the sample to the electrode array for measurement. A sample pretreatment consists of a 1 to 2 \(\mu\)m filter or small capillary silica column to remove particulate matter from the sample. This type of pretreatment will be integrated into the sample delivery tubing of the microfluidic channel. Treatment of the microchannel with a hydrophilic compound (such as a hydroxysilane) results in aqueous solutions being drawn into the device by capillary action. Development of a sampling head first requires a determination of the biological fluid (such as blood) to be used, and the necessary safety concerns (such as blood-born pathogens) to be addressed. A study with different types of filtration methods (paper, membrane, porous matrix, chemical), followed by analysis of the filtrate for ion concentration by the surface based sensors described previously, will determine pretreatment conditions. The selected filtration technique will be miniaturized for integration into the device.

For the sensor platform to be used as a multi-analyte detection device, an electrode array with an
independently addressable electrode for each analyte is required. The prototype described in this chapter uses a commercially available array with two active sites. A new array with at least 3 active regions (one for each crown ether) is envisioned. Electrode arrays are fabricated in a multistep process. The design is created with a software package (AutoCAD) that is used to make a photolithographic mask of the electrode array. A glass substrate is spin coated with a photoresist and exposed to light through the photomask, which polymerizes the resist in the exposed areas. The remaining resist is etched off the substrate, and an adhesion layer of chromium followed by a layer of gold is evaporated onto the substrate in the array pattern. The remaining resist is removed and the non-active areas of the array are passivated with a silicon nitride layer to generate the multi-electrode array.

Impedance spectroscopy measurements are performed on a potentiostat/frequency response analyzer interfaced with a PC, which is not suitable for remote or handheld monitoring. A handheld device (similar to a PDA) is needed to house the electronics required for the electrochemical measurement. An impedance measurement that used a single frequency or only a small number of frequencies, rather than the range of frequencies used for preliminary studies (25), is easier to implement in a handheld electronic device. Fewer frequencies also decrease the measurement time of the sensor. A study that examines how many and which particular frequencies are needed for accurate analyte binding measurements will assist in designing a handheld impedance analyzer for this device. The frequency range will be decreased from both the high and low end to determine how many points are required to accurately model the impedance data. Comparisons between the resistive and capacitive values derived from measurements with fewer points to those with a full frequency range (10 kHz to 100 mHz) will
determine how many individual frequencies are to be implemented into the portable detection system.
EXPERIMENTAL DETAILS

Materials and Methods.

General experimental methods and chemical supplier information is provided in Chapter 4. 1-Aza-12-crown-4, 1-aza-15-crown-5, and 1-aza-18-crown-6 were from Aldrich (Milwaukee, WI). Glucose oxidase (from Aspergillus Niger, type II-S, activity 15,000-50,000 units/g) (GOx) and D-(+)-glucose (99.5 %) were from Sigma (St. Louis, MO).

Monolayer Preparation.

SAMs were deposited on piranha cleaned gold substrates (as previously described) from 1 mM ethanolic solutions of the respective crown ether thiol analogue for 18 hours. Prior to characterization, substrates were removed from the deposition solution, rinsed with ethanol, and dried in a stream of nitrogen.

Microelectrodes.

Electrochemical Cell-On-A-Chip (ECCs) arrays were purchased from Abtech Scientific, Inc. (Richmond, VA). The microfabricated electrode arrays are deposited on borosilicate glass with dimensions of 20.0 ×10.0 × 0.50 mm. The ECC contains two active areas of 20 µm with 20 µm spacing between each of the interdigitated working electrodes of the array. Independently addressable reference and counter electrodes are also included. Electrodes consist of 1000 Å of evaporated gold on top of 100 Å of an evaporated titanium/tungsten adhesion layer. The inactive portion of the electrode array is coated with a silicon nitride passivation layer to insulate the gold leads. Connectivity to the instrument was accomplished with a test clip and adapter also from
Microelectrode Pretreatment.

Before chemical alteration the ECCs were cleaned by sonication in acetone for two minutes, rinsed with deionized water, and dried under nitrogen. Following cleaning microelectrodes were treated with oxygen plasma for 30 seconds in a SPI Supplies (West Chester, PA) Plasma Prep II plasma cleaner.

Silverization of Reference Electrode.

Silver was plated onto the reference electrode of the ECC to generate a stable reference for microfluidic measurements. The active area of the electrodes was immersed in a Techni-Silver Cy-less II Ready-to-Use electroplating solution (Technic, Inc., Cranston, RI). The reference microelectrode (to be plated) was set as the working electrode with a KCl saturated Ag/AgCl reference and a platinum counter electrode. The current \( I \) and time \( t \) for silverization was calculated using the working electrode area \( A \) and the charge density of 6456 mC/cm\(^2\) at a current density of 5.38 mA/cm\(^2\) by equations:

\[
I = A \times 5.38
\]

\[
t = \frac{A \times 6456}{I} = A \times 6456 \times \frac{1}{5.38} = 1200 \text{ s}
\]

After plating the ECC was rinsed with deionized water and dried under nitrogen.
**SAM Deposition on Microelectrodes.**

Following silver plating, microelectrodes were immediately placed in 1-2 mM solutions of azacrown thiols (12-crown-4, 15-crown-5, or 18-crown-6) in ethanol overnight. Electrodes were removed from the deposition solution, rinsed with ethanol, and dried with nitrogen before use.

**Glucose Oxidase Deposition on Microelectrodes.**

The ECC was chemically modified with glucose oxidase (GOx) in a three-step process. First a SAM of mercaptosuccinic acid (MSA) was formed on the surface by placing the electrode in a 20 mM aqueous solution overnight. Following monolayer deposition, the electrode was rinsed with deionized water and placed in a EDC/NHS activation solution for 30 min. The solution contained 0.002 M EDC and 0.005 M NHS in 0.1 M pH 5.5 phosphate buffer (prepared from 0.05 M K$_2$HPO$_4$ with 0.05 M KCl and 0.05 M KH$_2$PO$_4$ with 0.05 M KCl). The ECC was removed from the activation solution, rinsed with pH 5.5 buffer and placed in a 500 µg/mL GOx solution in 0.1 M pH 7.0 phosphate buffer (prepared with the same solutions described above) for 2.5 hrs. Following GOx immobilization, the electrode was rinsed with pH 7.0 buffer, dried with nitrogen, and used for electrochemical measurements.

**Device Fabrication and Assembly.**

Microfluidic channels were fabricated from polydimethysiloxane (PDMS) (Sylgard 184 Silicone Elastomer Kit, Dow Corning, Midland, MI). PDMS was mixed per manufacturer’s instructions (10:1 base to curing agent by mass), placed under vacuum to remove all air pockets, cast into a mold, and cured for 2-3 hours at 70 °C. The mold for the microfluidic channels was custom milled from solid aluminum with channel dimensions of 5 mm long x 0.5 mm wide x 1 mm
deep, with total dimensions of the cast PDMS of 12.7 mm x 6.4 mm x 6.3 mm. Following
curing, the PDMS microchannel was removed from the mold, briefly frozen in liquid nitrogen,
and small holes (~ 1.2 mm) were drilled at each end of the channel. Before the PDMS warmed
to room temperature, polyethylene tubing (I.D. 0.76 mm, O.D. 1.22 mm, Becton Dickinson,
Sparks, MD) was inserted into the holes at the end of each channel to allow for fluid delivery.
Once the PDMS warmed to room temperature and became flexible, a leak-free seal was obtained.

The PDMS microfluidic channel was fixed to the microelectrode array using a custom built
fixture fabricated from a 5 mm thick sheet of polypropylene. A recess was milled in the top
portion to accommodate the PDMS microchannel (12.7 mm × 6.4 mm × 6.3 mm), and a recess
was milled in the bottom portion to hold the ECC (20 mm × 10 mm × 0.5 mm). The two halves
of the fixture were held together with thumb screws and provided a way to assure the correct
channel/electrode alignment and also a leak-free system. The fixture also accommodated
connection of the test clip to the end of the ECC.

**Glucose Measurements.**

Impedance measurements were made using a three electrode setup as described previously.
Measurements were taken at the formal redox potential of p-benzoquinone (PBQ) (+ 0.28 V vs.
the reference) over a frequency range of 10 kHz to 100 mHz using either a 5 or 10 mV AC
perturbation. The electrolyte solution contained 5 mM PBQ and 0, 50, or 100 mM glucose in a
0.1 M pH 7.0 phosphate buffer (prepared from 0.05 M K₂HPO₄ with 0.05 M KCl and 0.05 M
KH₂PO₄ with 0.05 M KCl). Solutions containing glucose were prepared and stored at 4 °C.
overnight to allow for equilibration of the anomers, and PBQ was added immediately prior to the measurements.
SYNTHETIC DETAILS

4-mercapto-1-(1,4,7-trioxo-10-azacyclododecan-10-yl)butan-1-one.

1-Aza-12-crown-4 (0.95 g, 5.42 mmol) was combined with 1.2 equivalents of γ-thiobutyrolactone (0.665 g, 6.51 mmol) in the presence of 0.2 equivalents of camphorsulfonic acid (0.252 g, 1.08 mmol) in anhydrous toluene (30 mL) and heated with stirring at 100 °C for 2 days. The reaction was then diluted with anhydrous toluene (20 mL), and aqueous saturated sodium bicarbonate (50 mL) was added. The aqueous portion was extracted 3 times with dichloromethane, and the organic portions were combined, washed with water, dried over magnesium sulfate, and the solvent removed by rotary evaporation. The crude product was purified by silica gel column chromatography using dichloromethane/methanol (v/v 50:1) as eluent. Fractions containing the pure product (identified by TLC) were combined, the solvent was removed by rotary evaporation, and the product was dried under vacuum resulting in a yellow oil. Yield: 0.597 g (39.70 %). 1H–NMR (CDCl3) δ (ppm): 3.94-3.52 (m, 16 H, crown), 2.57 (m, 4 H, 2 CH2), 1.96 (q, 2 H, CH2), 1.38 (t, 1 H, SH). 13C–NMR (CDCl3) δ (ppm): 173.0, 71.7, 70.3, 69.8, 69.7, 69.6, 69.5, 50.6, 49.3, 31.4, 29.4, 24.4.
**4-mercapto-1-(1,4,7,10-tetraoxa-13-azacyclopentadecan-13-yl)butan-1-one.**

1-Aza-15-crown-5 (2.0 g, 9.12 mmol) was combined with 1.2 equivalents of γ-thiobutyrolactone (1.12 g, 10.94 mmol) in the presence of 0.2 equivalents of camphorsulfonic acid (0.42 g, 1.82 mmol) in anhydrous toluene (40 mL) and heated with stirring at 100 °C for 2 days. The reaction was diluted with anhydrous toluene (20 mL), and aqueous saturated sodium bicarbonate (50 mL) was added. The aqueous portion was extracted 3 times with dichloromethane and the organic portions were combined, washed with water, dried over magnesium sulfate, and the solvent removed by rotary evaporation. The crude product was purified by silica gel column chromatography with dichloromethane/methanol (v/v 50:1) as the eluent. Fractions containing the pure product (identified by TLC) were combined, the solvent was removed by rotary evaporation, and the product was dried under vacuum resulting in a yellow oil. Yield: 0.504 g (17.19 %). \(^1\)H–NMR (CDCl\(_3\)) \(\delta\)(ppm): 3.82-3.54 (m, 20 H, crown), 2.60 (q, 2 H, CH\(_2\)), 2.51 (t, 2 H, CH\(_2\)), 1.95 (q, 2 H, CH\(_2\)), 1.34 (t, 1 H, SH). \(^{13}\)C–NMR (CDCl\(_3\)) \(\delta\)(ppm): 172.5, 71.6, 70.6, 70.3, 70.2, 70.1, 69.8, 69.6, 50.4, 49.4, 31.3, 29.3, 24.3.
4-mercapto-1-(1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl)butan-1-one.

1-Aza-18-crown-6 (2.0 g, 7.60 mmol) was combined with 1.2 equivalents of γ-thiobutyrolactone (0.93 g, 9.11 mmol) in the presence of 0.2 equivalents of camphorsulfonic acid (0.35 g, 1.52 mmol) in anhydrous toluene (40 mL) and heated with stirring at 100 °C for 2 days. The reaction was diluted with anhydrous toluene (20 mL), and aqueous saturated sodium bicarbonate (50 mL) was added. The aqueous portion was extracted 3 times with dichloromethane and the organic portions were combined, washed with water, dried over magnesium sulfate and the solvent removed by rotary evaporation. The crude product was purified by silica gel column chromatography using dichloromethane/methanol (v/v 50:1) as the eluent. Fractions containing the pure product (identified by TLC) were combined, the solvent was removed by rotary evaporation, and the product was dried under vacuum resulting in a yellow oil. Yield: 0.250 g (9.01 %). $^1$H–NMR (CDCl$_3$) δ(ppm): 3.71-3.55 (m, 24 H, crown), 2.60 (q, 2 H, CH$_2$), 2.52 (t, 2 H, CH$_2$), 1.95 (q, 2 H, CH$_2$), 1.35 (t, 1 H, SH). $^{13}$C–NMR (CDCl$_3$) δ(ppm): 172.4, 70.1, 70.8, 70.7, 70.6, 70.4, 70.0, 69.5, 49.0, 46.9, 31.2, 29.3, 24.4.
s-Acetyl-3-mercaptopropanol.

A 500 mL flask was equipped with a condenser and two addition funnels. Allyl alcohol (130 g, 2.24 mol) was added to the flask. One of the addition funnels was charged with thioacetic acid (130 g, 1.71 mol) and to the other a solution of 2,2′-azoisobutyronitrile (AIBN) (1.8 g, 10.96 mmol) in 25 mL allyl alcohol was added. Under nitrogen, with vigorous stirring, a slow co-feed of the acid and initiator was started; addition was complete in 30 minutes. The reaction mixture was stirred for 4 hours at room temperature and the solution turned pink to light red in color. Excess alcohol was removed under reduced pressure and the crude product was purified by vacuum distillation yielding a yellow liquid (55 mL collected). $^1$H–NMR (CDCl$_3$) δ(ppm): 3.64 (t, 2H, O–CH$_2$), 2.99 (t, 2H, S–CH$_2$), 2.81 (s, 1H, OH), 2.35 (s, 3H, CH$_3$), 1.82 (q, 2H, CH$_2$). $^{13}$C–NMR (CDCl$_3$) δ(ppm): 197.1, 60.4, 32.4, 30.6, 25.5.
A 500 mL 4-neck flask was equipped with 2 addition funnels and a calcium sulfate drying tube. The flask was charged with oxalylchloride (9.46 g, 74.51 mmol) in 100 mL anhydrous dichloromethane. With vigorous stirring, the flask was cooled to – 78 °C. Dimethylsulfoxide (DMSO) (11.64 g, 149.03 mmol) was added to one addition funnel and s-acetyl-3-mercaptopropanol (5.0 g, 37.26 mmol), in 50 mL anhydrous dichloromethane, was added to the other. DMSO was slowly added to the reaction flask and allowed to mix for 10 minutes, followed by slow addition of the alcohol and 15 minutes of stirring. A portion of triethylamine (TEA) (15.08 g, 149.03 mmol) was then slowly introduced to the reaction mixture and allowed to stir for 10 minutes, followed by allowing the reaction to warm to room temperature and an additional 10 minutes of stirring. A 150 mL portion of water was added, the organic layer was washed with brine, and dried over magnesium sulfate. The solvent was removed under reduced pressure and the crude product was purified by vacuum distillation yielding a dark yellow liquid.

\(^1\)H–NMR (CDCl\(_3\)) \(\delta\) (ppm): 9.75 (s, 1H, OCH), 3.12 (t, 2H, \(\text{CH}_2\)), 2.81 (s, \(2\text{H, S-CH}_2\)), 2.34 (s, \(3\text{H, CH}_3\)). \(^{13}\)C–NMR (CDCl\(_3\)) \(\delta\) (ppm): 199.9, 195.5, 43.7, 30.5, 21.5.
Tert-butyl 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7-carboxylate.

A sample of 4,13-diaza-18-crown-6 (5.96 g, 22.70 mmol) was suspended in dioxane (130 mL) and heated with stirring at 40°C to completely dissolve. A solution of 4.97 g (22.70 mmol) di-tert-butyl dicarbonate in dioxane (70 mL) was slowly added. Stirring was continued at 40°C for 30 minutes, followed by stirring at room temperature overnight resulting in a light yellow solution. The reaction was concentrated by rotary evaporation and diethyl ether (30 mL) was added to precipitate any unreacted diazacrown starting material, which was removed by vacuum filtration. The filtrate was concentrated by rotary evaporation and the crude product was purified by column chromatography (alumina) using dichloromethane/methanol (v/v 200:1) as eluent. The product was identified by TLC, the solvent removed by rotary evaporation, and the product was dried under high vacuum resulting in a yellow oil. Yield: 2.34 g (28.5%). $^1$H-NMR (CDCl$_3$) δ (ppm): 3.60 (m, 16H, crown), 3.51 (t, 4 H, NCH$_2$), 2.79 (t, 4H, NCH$_2$), 2.15 (s, 1H, NH), 1.45 (s, 9H, CH$_3$). $^{13}$C NMR (CDCl$_3$) δ (ppm): 155.5, 79.4, 70.5, 70.4, 70.3, 70.2, 70.1, 70.0, 69.7, 67.1, 49.4, 47.6, 47.3, 28.5.
Tert-butyl 16-(anthracen-9-ylmethyl)-1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7-carboxylate.

A solution of 9-chloromethyl anthracene (0.537 g, 2.37 mmol) and tert-butyl 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7-carboxylate (0.85 g, 2.37 mmol) in butyronitrile (100 mL) was heated at reflux for 2 days in the presence of 5.02 g (47.31 mmol) sodium bicarbonate and 0.052 g (0.311 mmol) potassium iodide. The reaction was cooled, filtered by vacuum filtration, and the filtrate concentrated by rotary evaporation. The residue was dissolved in dichloromethane (25 mL) and washed 3 times with water, dried over magnesium sulfate, concentrated by rotary evaporation, and dried under vacuum yielding a thick yellow/orange oil. Yield: 1.23 g (94.9 %). A small amount of solvent (butyronitrile) remained as it could not be removed under high vacuum. $^1$H-NMR (CDCl$_3$) $\delta$(ppm): 8.54 (d, 2H, anth), 8.39 (s, 1H, anth), 7.97 (d, 2H, anth), 7.49 (t, 2H, anth), 7.44 (t, 2H, anth), 4.59 (s, 2H, CH$_2$-anth), 3.60-3.50 (m, 20H, crown), 2.90 (m, 4 H, crown), 1.45 (s, 9H, CH$_3$). $^{13}$C NMR (CDCl$_3$) $\delta$ (ppm): 155.5, 134.4, 131.4, 130.4, 128.9, 127.4, 125.5, 125.2, 124.8, 79.4, 70.8, 70.7, 70.5, 70.4, 70.3, 70.1, 70.0, 53.9, 53.7, 53.4, 48.2, 48.0, 28.5.
7-(Anthracen-9-ylmethyl)-1,4,10,13-tetraoxa-7,16-diazacyclooctadecane.

A 1.26 g (2.28 mmol) portion of previously prepared tert-butyl 16-(anthracen-9-ylmethyl)-1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7-carboxylate was dissolved in dichloromethane (10 mL). While stirring, trifluoroacetic acid (2 mL) was slowly added dropwise. Stirring was continued at room temperature overnight resulting in a dark brown solution. The product was isolated by rotary evaporation and the resulting thick oil was dissolved in dichloromethane (25 mL) and washed with 10% aqueous sodium bicarbonate (25 mL) three times. The organic portion was dried over magnesium sulfate, the solvent removed by rotary evaporation, and the product dried under vacuum resulting in a thick brown oil. Yield: 0.92 g (89.2%).

$^1$H-NMR (CDCl$_3$) $\delta$(ppm): 8.57 (d, 2H, anth), 8.38 (s, 1H, anth), 7.97 (d, 2H, anth), 7.48 (t, 2H, anth), 7.44 (t, 2H, anth), 4.61 (s, 2H, CH$_2$-anth), 3.60-3.56 (m, 16H, crown), 2.90 (t, 4H, crown), 2.80 (t, 4H, crown).

$^{13}$C NMR (CDCl$_3$) $\delta$ (ppm): 134.1, 131.4, 130.7, 129.3, 129.1, 128.8, 127.3, 127.2, 125.7, 125.5, 125.3, 124.8, 123.5, 71.0, 70.5, 70.3, 70.1, 53.7, 53.4, 52.0, 49.3.
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