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SURFACE MODIFICATION WITH SELF-ASSEMBLED MONOLAYERS
FOR IMMUNOSENSOR APPLICATION

by

YONGWOO LEE

Submitted in partial fulfillment of the requirements for the Degree of
Doctor of Philosophy

Thesis Advisor:
Professor Chaim N. Sukenik

Department of Chemistry
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August 1995
CASE WESTERN RESERVE UNIVERSITY

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(chair)  

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Jan. 1, 1995

Steinberg

Chang Shu

date July 6, 1995

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SURFACE MODIFICATION WITH SELF-ASSEMBLED MONOLAYERS
FOR IMMUNOSENSOR APPLICATIONS

Abstract

by

YONGWOO LEE

The syntheses and characterization of long-chain alkyltrichlorosilanes of alkyl halides, benzyl halides, and α-haloacetyl derivatives designed to form siloxane-anchored self-assembled monolayers (SAMs) for the selective attachment of peptides through cysteiny1 thiol groups is described. Thin film formation by the trichlorosilanes was demonstrated by Electron Spectroscopy for Chemical Analysis (ESCA) and by surface wetting properties. Halide exchange could be utilized to produce the more reactive iodide-bearing surface in situ, from the more stable chloride or bromide-bearing surfaces. In solution, these functional groups were found to have a range of reactivity with model thiols which extended from half-lives of minutes to days. The order of reactivity is I > Br > Cl within each class of compounds, and α-haloacetyl > benzyl >> alkyl. The reactivity of the SAMs with thiols showed the same order of reactivity. The very reactive α-iodoacetyl SAM was also reactive with amines, but competition experiments demonstrated preference for the thiol under our reaction conditions. SAM reactivity with cysteine-containing peptides was demonstrated with a tripeptide (glutathione) and a nonapeptide (laminin fragment). The attachment was completely blocked by prior treatment of these peptides with dinitrophenylmaleimide or by air oxidation of the thiol. These selective blocking experiments indicate that these SAMs will be useful for the directed attachment (through cysteine side chains) of proteins and
peptides. Based on these preliminary results of peptide attachments, Human Immunodeficiency Virus (HIV) antigen was attached to the α-iodoacetyl SAM under the same conditions. Its antigenic activity on the SAM surface was assessed by Enzyme Linked Immunosorbent Assay (ELISA) and by immunogold labeling.
To my mother, Kwangsoon Han, who sacrificed her entire life for her children
I would like to express my deepest gratitude to Professor Chaim N. Sukenik, my research advisor, and Professor James E. Zull for their patience and encouragement. Throughout my study Professor Sukenik always acknowledged my ideas with respect and added his views with sincerity. Such an unwavering understanding and support are rare qualities where I come from. I will always be in awe of that. As for Professor Zull, his desire to assist me in every way has my undying gratitude.

I would also like to thank all those professors, colleagues and friends who gave up enormous amount of time and energy for this thesis: My thesis committee -- Dr. Klippenstein, Dr. R. G. Salomon, and Dr. L. M. Sayre; Dr. W. Jennings, Professor C. C. Liu, Dr. Stan Prybyla, Dr. J. Reed-Mundell, Dr. M. Sandifer, Professor R. F. Savinell, Don Cameron, Rochael Collins, Beatrice Lin, Chris, Dmitri, Jeff, Moorthy, Maheshi, Vilnis, Gwang-Hoon, Kwang-Sun and Professor Kwang-Man Lee (Jeju National University in Korea). Without each and every one of their contributions, this thesis would not have been possible.

My love and thanks go to my mother, Kwang-Soon Han, and my sisters, Young-Sook and Jung-Soo Lee. Their incredible faith in me not only had given birth to my dreams, but also gave me the strength to finish.

Also, my special appreciation goes to Mr. & Mrs. Jung-Hun Choi who supports me in every aspect, and my "support group" in Korea -- V.O.G.M. (Choon-Hee Ryu who has served our Lord as a staff of VOGM with unselfishness, Hyun-Chul Kim as president and Nam Soo who has launched "Voice of Gospel and Mind", Yoon-Jeong
Kwon, Eui-Kyung Park who has established "Gospel Image Group" with the love of God and Sharon Ballet (Hye-Young Kim) -- for the glory of God.

Finally, I would like to give my special love and affection to Lauren Sun-Young Woo without whom I could not have maintained my sanity.

Yongwoo Lee
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>ABPS</td>
<td>2,2'-Azino-bis(ethylbenzthiazoline-6-sulfonic acid) diammonium salt</td>
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<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>APS</td>
<td>3-Aminopropyltriethoxysilanes</td>
</tr>
<tr>
<td>ATR/FTIR</td>
<td>Attenuated total reflectance Fourier transform infrared spectroscopy</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BSC</td>
<td>Benzenesulfonyl chloride</td>
</tr>
<tr>
<td>CA</td>
<td>Contact angle</td>
</tr>
<tr>
<td>Dansyl</td>
<td>1-Dimethylaminonaphthalene-5-sulfonyl chloride</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCH</td>
<td>Dicyclohexyl</td>
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<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DT</td>
<td>1-Decanethiol</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithio-bis(2-nitrobenzoic acid)</td>
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<tr>
<td>EA</td>
<td>Ethyl acetate</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
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<td>EMCS</td>
<td>N-succinimidy 6-maleimidocaproate</td>
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<td>ESCA</td>
<td>Electron spectroscopy for chemical analysis</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>FAB</td>
<td>Fragment of antibody</td>
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<tr>
<td>FTIR</td>
<td>Fourier transform infra-red</td>
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<td>GID</td>
<td>Grazing incidence diffraction</td>
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<td>GS-SG</td>
<td>Oxidized glutathione</td>
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<tr>
<td>H</td>
<td>Hysteresis</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HOPG</td>
<td>Highly oxidized pyrolytic graphite</td>
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<tr>
<td>HPLC</td>
<td>High performance (pressure) liquid chromatography</td>
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<tr>
<td>HTS</td>
<td>Hexadecyltrichlorosilane</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>L-B film</td>
<td>Langmuir-Blodgett film</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>NEM</td>
<td>N-ethyl maleimide</td>
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<tr>
<td>NLO</td>
<td>Non-linear optics</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>ODT</td>
<td>Octadecanethiol</td>
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<tr>
<td>OTE</td>
<td>Octadecyltriethoxysilane</td>
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<tr>
<td>OTMS</td>
<td>Octadecyltrimethoxysilane</td>
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<tr>
<td>OTS</td>
<td>Octadecyltrichlorosilane</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<td>PEO</td>
<td>Polyethylene oxide</td>
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<td>PNTP</td>
<td>p-Nitrothiophenol</td>
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<tr>
<td>QCM</td>
<td>Quartz crystal microbalance</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<td>SAW</td>
<td>Surface acoustic wave</td>
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<td>SAM</td>
<td>Self-assembled monolayer</td>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
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<td>SFG</td>
<td>Sum frequency generation</td>
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<td>SIMS</td>
<td>Secondary ion mass spectrometry</td>
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<tr>
<td>SHG</td>
<td>Second harmonic generation</td>
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<td>STM</td>
<td>Scanning tunneling microscopy</td>
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<td>(n)-Tricontyltrichlorosilane</td>
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<tr>
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<td>Transmission electron microscopy</td>
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<td>THF</td>
<td>Tetrahydrofuran</td>
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<td>TLC</td>
<td>Thin layer chromatography</td>
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<td>UHV</td>
<td>Ultra high vacuum</td>
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<td>XAFS</td>
<td>X-ray absorption fine structure</td>
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<td>X-ray photoelectron spectroscopy</td>
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Surface Modification with Self-Assembled Monolayers for Immunosensor Applications

Chapter 1. Introduction: Self-Assembled Monolayers

1-1. Background

Organized molecular assemblies, known as Self-Assembled Monolayers (SAM), have attracted the attention of many researchers in recent years. Since they provide monomolecular thin films simply by dipping solid substrates into a homogeneous solution, this technology has been examined as an alternative and/or a complementary tool to Langmuir-Blodgett films for application in the areas of microelectronics, optoelectronics, wear protection, and biosensing devices, etc. In addition, the ability to control the molecular order and packing in 2-dimensions makes them well suited for fundamental understanding of various phenomena including lubrication, adhesion, corrosion, diffusion and crystal growth.

Figure 1-1. Spontaneous adsorption of self-assembled monolayers. The organic molecular monolayer assembly is formed by a specific interaction between the head group (□) and the surface of the solid substrate.
Self-assembled monolayers form spontaneously when surface active organic molecules adsorb onto substrates by immersion (Figure 1-1). Even though chlorosilanes have been commonly used as surface modifiers since the 1940's, they first attracted attention as a potentially usable thin film technology when Sagiv\(^9\) reported the ultra-thin, uniform, siloxane-anchored monolayer, created by the assembly of octadecyltrichlorosilane (OTS). Since then, SAMs have emerged as the subject of many studies in a growing number of fields.

Ordered organic surfaces have been designed to achieve well-defined, organized assemblies at the molecular level. Langmuir-Blodgett films have been used for controlled film construction for many years. They were the first technique to provide ordered molecular assemblies. However, in spite of their uniformity and compact structure, L-B films have serious disadvantages in that they require carefully controlled experimental conditions and, once obtained, the films are very fragile. In contrast, siloxane anchored monolayers do not require any special facilities for preparation, and they are often robust enough to survive chemical and thermal stresses due to the covalent bonding and the \textit{in-situ} polymerized polysiloxane backbone. Moreover, they are closely packed, well-ordered, and nearly defect-free films, comparable to those obtained from the Langmuir-Blodgett film technique (Table 1-1).

To date, SAMs have been extensively used as surface modifiers or templates in many areas which require well-defined ultra-thin surface, e.g. biotechnology and materials science.\(^{10}\)
### Langmuir-Blodgett Film vs SAM Film

<table>
<thead>
<tr>
<th>L-B</th>
<th>SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Well-ordered</td>
<td>• Well-ordered</td>
</tr>
<tr>
<td>• Mechanically compressed</td>
<td>• Spontaneously adsorbed</td>
</tr>
<tr>
<td>• Fragile</td>
<td>• Robust</td>
</tr>
<tr>
<td>• Special equipment/conditions required</td>
<td>• No special equipment/condition needed</td>
</tr>
</tbody>
</table>

Table 1-1. Monolayer films. (special features: L-B film vs SAM film)

Stable SAMs can be attached with great integrity to various inorganic surfaces (e.g. Si wafers, SiO₂ glass slides, Ta₂O₅, Ti wafers, etc.). The deposition process can tolerate a range of pendant functionality, and chemical transformation can be achieved within the monolayer environment without any significant damage to the film. Several types of organized molecular assemblies are available for surface modification. These consist of organosilicon compounds on hydroxylated surfaces (Si / SiO₂, Al / Al₂O₃, Ti / TiO₂, Ta / Ta₂O₅, etc.), alkyl on Si,¹¹ alkanethiols or dialkyl disulfides on gold, copper, silver,¹² and mercury,¹³ aromatic thiol on gold,¹⁴ aliphatic alcohols or amines on Pt, aliphatic and aromatic carboxylic acids on aluminum oxide or silver,¹⁵ isocyanide derivatives on gold.¹⁶ Other variations of these systems include organosilicon (RSiCl₃) on highly oxidized pyrolytic graphite (HOPG)¹⁷ or on modified polymeric substrates (polydimethylsiloxane and polyethylene),¹⁸ alkanethiols on GaAs,¹⁹ and hydroxamic acids on TiO₂.²⁰
Figure 1-2. Components of self-assembled monolayer.

The structural relationships and molecular interactions of the SAM array are shown schematically in Figure 1-2. The molecular unit designated H is the anchoring head group functionality; the letter C represents the hydrocarbon chain; and the letter X represents the surface functionality that remains exposed after monolayer attachment.

1-2. Classification of the self-assembled monolayers

Many different kinds of bonding interactions have been shown to be useful for SAM formation (Figure 1-3). Alkyl or aryl carboxylates adsorb onto an Al₂O₃ surface through ionic bonding which greatly resembles Langmuir-Blodgett films in terms of
packing mode. Thiolates and disulfides strongly adsorb on gold, silver, or copper through electron transfer between the substrate surface and the SH functional group of the organic molecule. Organosilicon [RSiCl₃ or R(OSiMe₃)] compounds are covalently bound to hydroxylated substrates with added stability through cross-linking. Among these SAMs, the two most commonly used systems are the thiolate monolayer on gold and the siloxane-based monolayer on silicon.

![Diagram of bonding interaction of each SAM system. Siloxane-based SAM, thiolate anchored SAM, and carboxylate-anchored SAM.](image)

**Figure 1-3. Bonding interaction of each SAM system.** Siloxane-based SAM, thiolate anchored SAM, and carboxylate-anchored SAM.

1-2-1. Thiolate monolayer assemblies on gold or silver (R-SH and RS-SR)

Since Nuzzo\textsuperscript{21} \textit{et al.} found that dialkyl disulfides strongly adsorb on gold surfaces (Figure 1-4), thiolate monolayers\textsuperscript{22} on gold have been studied extensively.
Studies of formation of alkanethiolate monolayers on Au (111) demonstrate that at relatively dilute concentrations (10^{-3} M), two distinct adsorption kinetics can be observed. Approximately 80-90% of the surface is covered with monolayer within a few minutes, and the adsorption is complete within several hours to several days. In addition, long chain alkanethiols allow for faster adsorption kinetics than do the shorter ones, probably due to increased Van der Waals interactions between the interlayer chains.

![Diagram of alkanethiolate monolayers on Au (111). X = -CH_3, -OH, -COOH, -OCH_3, etc.](image)

Fig. 1-4. Thiolate monolayer assemblies on gold surface. Thiolate anchored monolayer assemblies to substrates are depicted.

As was mentioned earlier, the chemisorption of alkanethiols on a gold surface is known to occur by electron transfer from the gold surface to the thiol sulfur atoms. (eq. 1-1) Several studies\textsuperscript{23} have indicated that the S-H bond is cleaved at the on-top
site, and the thiolate then moves to the hollow site of the gold lattice. \(H_2\) evolution is the exothermic step in the chemisorption energetics.

\[
\begin{align*}
M-M \\
RS-H \quad &\longrightarrow \quad RS-M^+M^0 + 1/2 \text{H}_2 \\
M &\text{ = Au, Ag, Cu, etc.}
\end{align*}
\]

In order to elucidate the kinetics of thiolate SAM formation on gold, Karpovich\textsuperscript{24} et al. monitored \textit{in situ} mass change in real time of a quartz crystal microbalance (QCM) as it became coated with octadecanethiol (ODT). From those measurements, they found that the rate of monolayer formation strongly depends on monomer concentration. An attempt to probe the structural order of SAMs was made by Hines\textsuperscript{25} et al., who utilized Sum-Frequency Generation (SFG) to examine the conformation of thiolate SAMs on a variety of substrates. They suggested that alkyl thiolate monolayers on Au and Ag are well-packed and in an all-trans conformation. Chidsey\textsuperscript{26} et al. suggest that there exists a correlation between the terminal (non-anchoring) functional group and the defects present, showing that fluorinated alkanethiols are the least defective while the carboxylic acid-terminated monolayers are the most defective among various thiolate SAMs. Chailapakul\textsuperscript{27} et al. demonstrated using STM that the deposition solvent and chain length affected the packing and order of thiolate SAMs. Bain\textsuperscript{28} et al. reported that the degree of order on the SAM surface can be manipulated by controlling the composition of the monolayers. Offord\textsuperscript{29} et al. attempted to probe the factors that control monolayer formation in disulfide systems with the use of XPS, ellipsometry, and TOF-SIMS techniques and showed a dramatic effect of chain length on packing efficiency. In their report, the longer chain species provide well-packed monolayers while the shorter chains do not. Also, SAMs which
were formed from equal amounts of mixed dialkyl disulfides exhibited preferential adsorption of the longer-chain species. Schneider\textsuperscript{30} \textit{et al.} studied the correlation between the deposition solvent and formation kinetics using an electrochemical quartz crystal microbalance (EQCM). From those experiments, they suggested that the deposition conditions greatly influence the quality of the SAM.

Based on the fundamental understanding of thiolate SAMs on Au surfaces summarized above, thiolate-monolayer assemblies on surfaces have been used in many applications.\textsuperscript{31} An octadecanethiol (ODT) monolayer can serve as a well-defined electron transfer barrier on a gold electrode.\textsuperscript{32} A carboxylate-terminated thiolate SAM was employed in a surface acoustic wave-based (SAW) biosensor\textsuperscript{33} monitoring organophosphate nerve agent stimulants. Abbott\textsuperscript{34} \textit{et al.} demonstrated that the wettability of surfaces could be manipulated using hydrophilic and hydrophobic thiolate SAMs on a micromachined gold surface, proposing that it can serve as a good alternative to microlithography. A monolayer of nickel hexacyanoferrate\textsuperscript{35} was achieved (based on carboxylate-terminated sulfide monolayer on gold) which was capable of recognizing Na\textsuperscript{+} and K\textsuperscript{+} ions. Photoisomerizable glucose oxidase monolayers\textsuperscript{36} could be obtained based on NH\textsubscript{2}-terminated thiolate monolayers on gold. Ferroceneoctanethiol monolayers\textsuperscript{37} were studied on metal-coated high-temperature superconductor electrodes at Sub-Tc temperatures. The use of thiolate monolayers on gold systems for photo-patterning is also seen in a number of reports.\textsuperscript{38}

Despite the interest in these films, the thiolate SAMs on gold have limitations. A recent report demonstrated that \textit{n}-alkanethiols initially chemisorbed on an Au (111) surface can be desorbed from the surface by an AFM tip.\textsuperscript{39} It was found that even strongly bound monolayer molecules could be displaced under certain loads. This means that the arrangement of the molecules on the surface, in some cases, can be
physically altered in a controlled manner by applying a certain amount of force. The thiolates adsorbed on gold have also been found to be unstable in the presence of alkaline solutions.\textsuperscript{40} Also the C-S bond cleavage of thiolate monolayers was observed by electrochemical measurements.\textsuperscript{41} It has been also revealed, with the help of STM, that defects are present in the thiolate monolayer surfaces.\textsuperscript{42-43} In addition, within the electrochemical environment, the gold surface under the thiol has been found to be dissolved.\textsuperscript{44} In light of these results, it is clear that thiolate monolayers are not robust enough to survive chemical and electrochemical manipulation and still retain their microstructure, particularly when compared to siloxane-based monolayer assemblies.

1-2-2. Siloxane-based monolayer assemblies (RSiCl\textsubscript{3})

Siloxane-based molecular SAMs are uniform, stable, well-packed, ultra-thin films that form spontaneously on the surface, and are known to have thermal and chemical stability under various conditions due to their cross-linked polysiloxane backbone (Figure 1-5). Maoz\textsuperscript{45} et al. have performed comprehensive studies on various monolayer assemblies on different substrates in an attempt to improve the basic understanding of the formation and structure of siloxane-based SAMs. They suggested that SAMs are structurally indistinguishable from LB monolayers of the same or similar compounds on a surface. In order to further elucidate the physical and chemical properties of SAMs, the octadecyltrichlorosilane (OTS) SAM has been chosen for many studies. Various analytical tools such as X-ray reflection and grazing incidence diffraction (GID),\textsuperscript{46} polarized ATR-FTIR,\textsuperscript{47} AFM, contact angle,\textsuperscript{48} ellipsometry,\textsuperscript{48} ESCA,\textsuperscript{48} and XAFS\textsuperscript{49} have been employed to confirm that OTS films are strongly adsorbed, well-packed, organized monolayers.
The initial report\textsuperscript{50} of multilayer assemblies based on molecular self-assembly encouraged researchers to develop a comprehensive understanding of the self-assembled monolayer as a template for many applications. As a simple example, a sulfonate-terminated SAM was used as a very efficient template for TiO\textsubscript{2} thin film formation.\textsuperscript{51} The OTS SAM was studied as a potential lubricant to reduce friction in micromotors.\textsuperscript{52} Friction and wear properties of siloxane-based monolayers were studied to reveal the usefulness of siloxane-based organic thin films.\textsuperscript{53}

\[
\begin{align*}
\text{X} & \quad \text{X} & \quad \text{X} & \quad \text{X} & \quad \text{X} \\
& \quad \text{SiCl}_3 & \quad \text{SiCl}_3 & \quad \text{SiCl}_3 & \quad \text{SiCl}_3 \\
& \quad \text{OH} & \quad \text{OH} & \quad \text{OH} & \quad \text{OH} \\
& \quad \text{O} & \quad \text{Si} & \quad \text{O} & \quad \text{Si} & \quad \text{O} & \quad \text{Si} & \quad \text{O} & \quad \text{Si} & \quad \text{O} & \quad \text{Si} & \quad \text{O} & \quad \text{Si} & \quad \text{O} \\
\end{align*}
\]

Monolayer Deposition

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

\[X = \text{CH}_2\text{CH}_2\text{CH}_3, \text{CH}_2\text{Br}, \text{CH}_2\text{CN}, \text{etc.}\]

\textbf{Figure 1-5. Formation of siloxane-based SAMs.} Surface OH groups are exposed to trichlorosilanes for surface attachment.
The OTS SAM has been employed in many studies as a model SAM because its monolayer film is most easily prepared on a variety of solid substrates. It has been used in the preparation of reverse phase HPLC columns, lubricants, glass-reinforced composites,\textsuperscript{54} chemical sensors\textsuperscript{55} and biological studies,\textsuperscript{56} etc. Its molecular arrangement on the solid substrate has been probed by polarized FTIR/ATR and confirmed to be well-ordered.\textsuperscript{47} The adsorption kinetics of OTS, reported first by Sagiv,\textsuperscript{9} was investigated with the help of ATR / FTIR in an \textit{in situ} observation of OTS monolayer assembly.\textsuperscript{57} It was found to be consistent with OTS employing the surface hydroxyl groups for polymeric siloxane formation. The most commonly accepted mechanism is the three step process, which was first proposed by Sagiv (Scheme 1-1).

\[
\begin{align*}
\text{R-Si-Cl} \quad \longrightarrow \quad \text{R-Si-OH} & \quad \text{at the hydroxyl substrate surface (1)} \\
\text{R-Si-OH} \quad \cdots \text{HO-Si-(surface)} & \quad \text{physisorbed by hydrogen bonding (2)} \\
\text{Si-O-Si} & \quad \text{polysiloxane backbone by cross-linking (3)}
\end{align*}
\]

\textbf{Scheme 1-1. OTS deposition process.}

Another mechanism, proposed by Angst\textsuperscript{58} \textit{et al.} and supported by Kessel\textsuperscript{59} \textit{et al.}, is that the hydrolysis occurs in the bulk instead of at the hydroxylic surface, and that octadecylsilatriol moves to the substrate surface for cross-linkage in the 2nd step. Other reports\textsuperscript{60} demonstrated that silane coupling agents could be immobilized on gold and mica surfaces. They seem to argue against the above mechanisms because of the absence of surface OH-functional groups in those substrates. In addition to these reports, Wood\textsuperscript{61} \textit{et al.} reported a novel approach using Langmuir-Blodgett deposition of prepolymerized octadecyltriethoxysilane (OTE) on a mica surface. However,
Nakagawa\textsuperscript{62} et al. demonstrated that the OTS monolayer on mica surface was peeled off from the surface by the AFM tip at an applied force of 20 nN, suggesting that almost all the adsorbed OTS molecules are anchored to the mica surface not by covalent bonds but by physical adsorption. This, along with a solid state NMR study by Blumel\textsuperscript{63} confirms the original notion that the presence of hydroxyl or siloxane groups is a prerequisite for chlorosilane attachment.

Despite the many reports regarding covalent, siloxane-anchored monolayer formation, the deposition conditions are not well-defined and are still controversial. Hence, the deposition conditions used by various researchers are widely varied (e.g., in toluene at RT under 30% humidity\textsuperscript{47}; in a mixed solution of 20\% CCl\textsubscript{4} / Isopar-G for 30 min at RT\textsuperscript{58}; in mineral oil at RT for 2.5 h\textsuperscript{64}; in bicyclohexyl at RT for 30 min\textsuperscript{56}). It is agreed that the spontaneous adsorption is influenced strongly by the solvent used, the temperature, and the deposition time.

Regarding the choice of solvent, there have been many approaches. Sagiv\textsuperscript{65} proposed that the similarity between the solvent used (hexadecane) and the OTS hydrocarbon chain causes the incorporation of hexadecane into the OTS film. When Kallury\textsuperscript{66} et al. used hexadecane, voids were observed on the OTS SAM surface. McGovern\textsuperscript{67} et al. indicated that a longer hydrocarbon chain solvent is preferred for well-packed SAM formation (well-packed OTS monolayer formation in order of hexadecane = octane > hexane > pentane). They demonstrated that aromatic solvents (benzene and toluene) provide more densely packed OTS films than do other saturated hydrocarbon solvents. They suggested that benzene and toluene can solubilize the optimum quantity of water necessary for the formation of alkyltrisilanol species to polymerize onto the substrate surface.
Fujii et al. recently demonstrated that hexagonally close-packed OTS SAMs could be generated on an oxidized silicon surface only on prolonged exposure to the organic solution at low temperature (5 °C). They suggest that RT is not the best condition to achieve well-defined structure and that long-time deposited Si wafers provide the best packed structure. This result is consistent with the possibility that the trichlorosilanes could be hydrolyzed in the bulk, and organized on the surface by subsequent covalent bonding. Another recent report suggests that curing at high temperature (200 °C) completes the cross-linking process.

Ohtake et al. reported that the molecular arrangement in the SA monolayer was random or bulk-like as long as the carbon number of the hydrocarbon chain was less than eight, and that the monolayer was arranged along the surface of the substrate only when using chain lengths of eight or more. They strongly suggest that the short chain surfactant does not form a well-packed, uniform monolayer assembly.

In order to achieve a more comprehensive understanding of the correlation between order, chain length and terminal functional group in the chlorosilane monolayers, Bierbaum et al. employed X-ray Absorption Fine Structure Spectroscopy (XAFS) along with X-ray Photoelectron Spectroscopy (XPS). Their findings suggest that NH₂-terminated silane films, regardless of the hydrocarbon chain length, are completely disordered, probably due to the interaction of the NH₂ group with the surface, whereas OTS tends to form self-assembled monolayers which are oriented almost perpendicular to the surface. The short chain silanes are randomly coiled and never form well-ordered monolayers. Alkyl trialkoxyisilanes (OTMS) are less well oriented than alkyl trichlorosilanes (OTS), suggesting the bonding might not be the same in these two systems. n-Triacontyltrichlorosilanes (TCTS), consisting of longer chain length than OTS, were shown to be less well oriented than OTS films,
suggesting that there should be an optimal chain length for ordering in siloxane-based organic monolayers.\textsuperscript{49}

1-2-3. Multilayers based on self-assembled monolayers

Since Langmuir-Blodgett films provide well-packed multilayers for application with clean lab facilities, it has been favoured for use in industrial applications. However, the incorporation of a molecular unit which does not allow for a close-packed structure into the L-B film can produce relatively disordered domains.\textsuperscript{71}

Decher\textsuperscript{72} et al. achieved multilayer assemblies by alternating the sequential physisorption of polyanionic chemical species (Figure 1-6). They utilized the polyanionic-polycationic interaction to formulate over 100 layer-thick multilayers comprised of polystyrenesulfonate (PSS) and poly(allylamine) hydrochloride (PAH). This method is very useful provided that the strong polyanionic interaction is available within the system. Mao et al.\textsuperscript{73} developed this method further with the use of photopolymerizable amphiphiles in order to obtain a higher degree of order by tightening the skeletal backbone of the multilayer system. Kunitake\textsuperscript{74} et al. applied this concept to the construction of layer-by-layer assembly of alternate protein and polyon ultrathin films for the development of protein-based electronic devices.

Mino\textsuperscript{75} et al. reported that multilayers could be constructed \textit{via} the cycling of chemical adsorption of long chain aliphatic compounds bearing trichlorosilane groups, and electron beam irradiation processing. However, the electron beam process severely damages the constructed multilayers, preventing it from providing well-defined multilayer structures. Mallouk\textsuperscript{76} et al. modified a gold surface with (4-mercaptobutyl)phosphonic acid and achieved a more than 100-layer thick multilayer by
alternating immersion into a 5 mM ethanolic solution of the metal acetate or perchlorate salt and $\text{H}_2\text{O}_2\text{P(CH}_2\text{nPO}_2\text{H}_2$, $n = 8, 10, 12, 14$.

![Diagram](image)

**Figure 1-6. Multilayer construction by physisorption of polyionic species.** (A) polycationic species: polyallylamine hydrochloride (PAH); (B) polyanionic species: polystyrenesulfonate (PSS).

Sagiv,\textsuperscript{50} who demonstrated OTS monolayer formation by spontaneous adsorption, developed a strategy for covalent multilayer construction with SAMs. The process resembles the classical L-B film methods except that it uses an *in situ* chemical transformation. The chemisorption of an olefin-terminated, bifunctional silane monolayer on a Si surface renders the modifiable functional group (the olefin) accessible at the monolayer surface. The olefin group can then be transformed into a hydroxyl functionality by hydroboration/oxidation. The OH terminal group created *in*
*situ* again allows for subsequent adsorption of the silane compound as a second layer from the original organic solution. This approach showed promise in the multilayer construction comparable to the classical L-B method, but was only demonstrated for very thin films (3 cycles).

A later attempt at multilayer build-up was made by Ulman, demonstrating that self-assembly could be an alternative to the classical L-B technique in the construction of relatively thick, ordered, multilayer films. In his approach, methyl 23-(trichlorosilyl) tridecanoate was initially deposited on a Si wafer by spontaneous adsorption. The generated organic -CO₂R (ester) surface was then reduced by the action of LiAlH₄ in ether solvent. The reduced organic surface bearing OH-functional groups then served as the template for the subsequent deposition of the surfactant by self-assembly. It was cycled repeatedly until almost a 0.1 µm scale thick organic superlattice was obtained. The synthetic modifications of surfaces that were used by Sagiv and by Ulman are shown in Figure 1-7.

The report by Sagiv was the first to achieve multilayer construction based on self-assembled monolayers. It opened the door to sophisticated film fabrication using synthetic modification of organic monolayer assemblies. Nevertheless, due to side reactions in the hydroboration / oxidation chemistry, non-homogeneous organic surfaces were created, hindering the sequential adsorption at each deposition process. The moisture-sensitive borane reagent makes it somewhat inconvenient to control the stoichiometry of the surface organic reaction and the oxidation could cause surface damage possibly via OH⁻ attack on the Si / SiO₂ surface. This problem is minimized by the route suggested by Ulman *et al*.. The long chain CO₂Me-terminated surfaces are very hydrophobic and form well-packed organized assemblies. LiAlH₄ was employed to obtain the OH-surface by reducing the ester function (CO₂R) of the SAM surface.
Micron scale thick films could be achieved during the cycling processes. In both methods, the creation of OH groups serving as the template for next layer deposition is crucial to multilayer construction, and the uniformity and accessibility of OH groups determines the packing density of the multilayers.

Figure 1-7. Comparison of the methodologies to the construction of the multilayer assemblies. (a) Sagiv's method $X = -CH_2=CH_2$, Reaction: hydroboration followed by oxidation. (b) Ullman's method $X = CH_2COOCH_2$, Reaction: LiAlH_4 reduction.
Another methodology for multilayer formation was introduced for Non-linear optics (NLO) materials, developed by Marks et al.. The chromophore-containing organic superlattice was obtained from the cycling of chemical transformation in three steps: (a) coupling layer formation (b) chromophore layer introduction (c) capping

![Diagram of multilayer build-up for NLO application](image)

**Figure 1-8. Multilayer build-up for NLO application.** The three step process which forms an NLO multilayer: (1) Hydrophobic electrophilic SAM (upper-left) was modified by the NLO moieties (arrow-inscribed). (2) OH-groups thus formed are capped by polysiloxane. (3) the deposition of initial monolayer assembly.
with polydimethoxysilane (PDMS) (Figure 1-8). High efficiency second harmonic generation (SHG) materials resulted from this methodology. These results demonstrate that self-assembly techniques represent a promising approach to the synthesis of thin film materials having high second order optical non-linearities.

Organic superlattice approaches using SAMs are greatly beneficial because of their potential use of self-assembly for electronics and Non-Linear Optics (NLO) applications. The idea that self-assembly resembles the classical L-B film construction is the impetus to the construction of such multilayers. The flexible and efficient functionalization of the organic surface is still the key issue for multilayer constructions.

1-3. Characterization of Organic Surfaces / Interfaces

The extensive use of organic surfaces in many fields such as biotechnology and materials science has stimulated rapid progress in the development of surface-sensitive analytical tools. There are a variety of instrumental techniques available for surface and interface analysis of monolayer assemblies bound to inorganic surfaces. General methods for surface and interface characterization of SAM are listed in Table 1-2.
<table>
<thead>
<tr>
<th>Techniques</th>
<th>Principles</th>
<th>Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact Angle Measurement</td>
<td>Predict the surface packing and its heterogeneity based on hydrophobic-hydrophilic relationships.</td>
<td>3-20 Å depth, surface energy measured.</td>
</tr>
<tr>
<td>Attenuated Total Reflectance FTIR (ATR / FTIR)</td>
<td>Monitor the vibrational mode to probe the presence of organic functional group</td>
<td>Not element specific, 0.5 cm to 20 μm, non-destructive</td>
</tr>
<tr>
<td>Electron Spectroscopy for Chemical Analysis (ESCA)</td>
<td>Probe all the atomic species except H and He</td>
<td>5-50 Å Depth, vacuum-compatible samples, semiquantitative analysis is possible.</td>
</tr>
<tr>
<td>Atomic Force Microscopy (AFM)</td>
<td>Monitor the cantilever deflection between the atoms on the surface and those on the tip.</td>
<td>Non-destructive Imaging and Mapping</td>
</tr>
<tr>
<td>Scanning Tunneling Microscopy (STM)</td>
<td>Measuring the quantum tunneling current between a metal tip and a conductive surface.</td>
<td>Non-destructive imaging and mapping. 5 Å depth</td>
</tr>
<tr>
<td>Secondary Ion Mass Spectroscopy (SIMS)</td>
<td>Monitoring the secondary ions emitted through the bombardment of a sample with an energetic beam of particles</td>
<td>100 μm, polymers characterization</td>
</tr>
<tr>
<td>Ellipsometry</td>
<td>Measure thickness and refractive index of organic thin films</td>
<td>Non-destructive, planar samples required</td>
</tr>
<tr>
<td>Scanning Electron Microscopy (SEM)</td>
<td>High magnification imaging</td>
<td>Must be coated with conducting films. 5 Å</td>
</tr>
</tbody>
</table>

Table 1-2. Commonly used surface analysis techniques

1-3-1. Contact angle measurement

The contact angle measurement is a classical technique that provides information about surface thermodynamic properties such as wetting and surface free energy and characterizes the solid surface itself. The angle formed between the liquid-vapour interface and the liquid-solid interface at the solid-liquid-vapour three phase contact line is defined as its contact angle. A contact angle on a solid can be defined in
a meaningful way only when a unique tangent plane to the surface can be observed objectively (Figure 1-9).

![Figure 1-9](image)

**Figure 1-9.** A sessile water drop on a solid surface. $\gamma_{SV}$, $\gamma_{SL}$, $\gamma_{LV}$: V: vapour phase, L: liquid phase. S: solid phase.

The three phase drawing shown in Figure 1-9, where a drop of liquid is in contact with a solid, shows the tangent angle defined between the second phase (liquid) and the third (gas). The type of system present in Figure 1-9 consists of three bulk phases: solid, liquid, and vapour and three interface phases: the solid-liquid, solid-vapour, and liquid-vapour interfaces. It allows us to deal with three surface tensions ($\gamma$): the conventional $\gamma_{LV}$ of the liquid-vapour interface, as well as $\gamma_{SV}$ and $\gamma_{SL}$, representing the solid surface tensions at the solid-vapour and solid-liquid interfaces, respectively. Though these solid surface tensions cannot be directly measured, they
can be estimated by interpreting the contact angle data. The difference in the surface free energy of the solid, $\gamma_{SV} - \gamma_{SL}$, can be deduced from the contact angle by the formula in eq. 1-2, where the surface tensions are related to $\theta$.

$$\gamma_{LV}\cos\theta = \gamma_{SV} - \gamma_{SL} \quad \text{(eq. 1-2)}$$

Hysteresis is another important phenomenon derived from the contact angle study. On all the liquids that form non-zero contact angles from the solid surface, hysteresis is observed and exploited in understanding the organic surface in terms of packing and order, provided that the liquid drops are considerably larger than the scale of the solid's heterogeneity and roughness. The hysteresis ($H$) of the wetting measurement—a reflection of the non-equilibrium nature of the contact angle measurements—can be defined as the difference between the advancing contact angle ($\theta_a$) and the receding contact angle ($\theta_r$) (eq. 1-3). It is inversely proportional to the liquid drop size on the surface. The value of the hysteresis obtained from the contact angle measurements is affected by the following four factors: heterogeneity of the solid surface, reorientation of the surface molecules on the surface, solubility of a surface component, and interaction between the surface and the liquid.

$$H = \theta_a - \theta_r \quad \text{(eq. 1-3)}$$

Marmur recently reviewed the importance of the contact angle and its dependence on the three-phase mutual interactions in the vicinity of the contact line. The usefulness of contact angle measurement for surface analysis was seen in a recent report regarding the wetting properties and stability of silane-treated glass slides.
exposed to water, air, and oil. It was demonstrated that the modified glass slides deteriorated as a function of time. The contact angle of the substrates was monitored carefully to determine the degree of deterioration. In another study, the interfacial tensions of a solid-liquid system were determined. The contact angles on heterogeneous surfaces were investigated by Israelachvili et al. to propose a simple model for a broad range of applications using contact angle measurements.

Contact angles play an important role in many dynamic processes. As with the solid surface, the use of contact angles for analyzing organic thin films allows us to qualitatively interpret the molecular packing and order of the given organic solid surface prior to the microscopic analysis for the detailed structural determination (Figure 1-10).

![Diagram of contact angles](image)

\[X = \text{Hydrophobic Group} \quad \text{X = Hydrophilic Group}\]

Figure 1-10. A sessile water drop on an organic SAM surface.

The qualitative interpretation, in turn, makes it possible to assess the quality of the organized assemblies in terms of packing and order, and orientation of the terminal
functional group of the monolayer within the 2-dimensional solid structure. It predicts
the surface reorientation caused by the hydrophobic-hydrophilic interaction at the film-
vapour interface.\textsuperscript{83} Whitesides\textsuperscript{84} et al. studied acid-base interactions on top of a
thiolate SAM surface by combining photoacoustic calorimetry and contact angle
titration. In addition, wetting property of an organic solid surface measured by contact
angle was investigated using sum-frequency spectroscopy in order to probe the
interaction between liquids and a SAM.\textsuperscript{85} Also, a systematic comparison of contact
angle methods was made recently by Lander\textsuperscript{86} et al.. They studied three different
techniques for measuring contact angles of monolayer assemblies: the Wilhelmy
balance, the tilting plate, and the sessile drop. For hexadecyltrichlorosilane (HTS)
monolayer adsorbed on silicon substrates, the Wilhelmy balance method proved to be
the best technique for measuring contact angle hysteresis reproducibly. They compared
their results to those obtained using the tilting plate technique and the sessile drop
method. Despite the fact that the Wilhelmy balance method gives more reproducible
results, the sessile drop method is still preferred for measuring the wetting properties
of an organic solid for surface characterization due to its simplicity and accessibility.
The acid-base behavior of monolayers assemblies were also studied by measuring
wetting properties of the surface as reported by Bain\textsuperscript{87} et al.. In their report, it was
shown that simple measurements of contact angles are enough to characterize the
chemically modified organic monolayer assemblies and even further monitor stepwise
chemical transformation of the mixed monolayer system due to high sensitivity to local
surface structure.
1-3-2. Electron Spectroscopy for Chemical Analysis (ESCA)

Electron Spectroscopy for Chemical Analysis (ESCA), also called X-ray Photoelectron Spectroscopy (XPS), has been extensively used in characterizing the monolayer surface. It is based on the photoelectric effect which was described by Einstein in 1905. The energetics of this process are described by eq. 1-4, where $E_b$ is the binding energy, $E_k$ is the kinetic energy of the emitted electron, $h\nu$ is characteristic energy of the x-rays, and $f$ is a constant.

$$E_b = h\nu - E_k - f \quad \text{(eq.1-4)}$$

When X-rays are focused upon a sample, the interaction of the x-rays with the atoms in the sample causes emission of core-level (inner shell) electrons, and the energy of electrons is measured by an energy analyzer (Figure 1-11).

![ESCA Instrument Diagram](image)

**Figure 1-11.** A schematic diagram of ESCA instrument.

ESCA provides unique information about the nature and environment of the atoms on the surface, which is unobtainable by contact angle measurements or by
surface microscopies. The following is a list of the types of information XPS can provide about the sample surface, as applied to functionalized organic SAMs.

- Detect all of the elements except H and He
- Monitor the appearance and disappearance of organic functional groups in a surface reaction
- Determine the presence of organic functional groups
- Differentiate the oxidation state and bonding information for multiple elements
- Analysis of reaction kinetics in surface chemistry

The data from ESCA (binding energy, peak shape, and intensity of the ESCA signal) can be interpreted in a simple but useful way. ESCA provides identification of all elements in the outermost 100 Å of a surface. It is commonly used to confirm and determine the composition of the surface and/or detect the presence of contaminants on the surface. Nuzzo et al. used ESCA to study the adsorption of dialkyl sulfides and alkanethiols on gold surfaces. Furthermore, thiolate monolayer assemblies were studied with ESCA to measure thickness based on the product \( \lambda K(T) \) of the inelastic mean free path and the inelastic scattering cross-section, which was previously measured for siloxane-based monolayers. An example of a very different kind of application is the kinetics study of the oxidation of iron and nickel surfaces. ESCA has been used to provide information about molecular bonding environments (i.e. methylolithium and dilithiomethane) and oxidation states of thin organic films. It was employed to examine the electronic structure of flavonoid compounds, demonstrating different oxidation states of each chemical species. Tilman et al. showed the presence of oxidized sulfur using ESCA, after a sulfur-
containing SAM was treated with H$_2$O$_2$. Kaneko\textsuperscript{97} et al. demonstrated that ESCA has the capability to determine the coverage of hydrophilic sites on hydrophobic surfaces and Kaushik\textsuperscript{98} reported the differentiation of oxidation states of Cu on a surface.

Angle-resolved ESCA provides compositional information by controlling the depth of penetration into the surface.\textsuperscript{99} An interesting experiment using angle-resolved ESCA measured the thickness of adsorbed proteins on a solid surface\textsuperscript{100} (Fig. 1-12). Subirade\textsuperscript{101} et al. demonstrated that angle-resolved ESCA could also provide information regarding the orientation of a globular protein on solid surfaces in addition to measuring the thickness of proteins adsorbed. A BSA adsorption study on a smooth mica surface was performed by ESCA to analyze the protein overlayer and a study of hemoglobin adsorbed on PTFE and platinum substrates. They showed that ESCA is also capable of monitoring the organization of the protein.\textsuperscript{102} The above results confirm the usefulness of ESCA for probing various systems in terms of thickness, kinetics study, and monitoring organic reactions. A combination of ESCA and contact angle measurements are the main tools used to study the surface transformations which will be discussed in the following chapters of this thesis.

Figure 1-12. Angle-resolved ESCA vs energy-resolved ESCA: Probing different domains by varying the energy strength or incident angle of the X-ray.
1-3-3. Various other methods for the structural analysis of the organic surfaces

As was described in the Table 1-2, there are a variety of methods available for the characterization and structure determination of self-assembled monolayers. A sampling of these methods is presented below.

ATR/FTIR has been routinely used to investigate the molecular packing and orientation in ultra-thin organic films. It also confirms the presence of various functional groups present within the organic thin film and can be used to monitor functional group transformations. Bertilsson\textsuperscript{103} et al. used ATR/FTIR to study mixed monolayers on gold and to prove that single-component domains or macroscopic islands do not form. Cheng et al. used ATR/FTIR to monitor the \textit{in situ} depositon of OTS.\textsuperscript{57} ATR/FTIR was also used to investigate the adsorption mode of fibronectin on variously coated Ge crystals.\textsuperscript{104} Bae\textsuperscript{105} et al. recently reported an \textit{in situ} ATR/FTIR study of molecular adsorbates at the electrode-electrolyte interface to provide information about interfacial electrochemical phenomena.

Ellipsometry is a surface analysis technique which measures layer thickness and refractive index. Due to its sensitivity and non-destructiveness, it has been favored for use in thin film analysis.\textsuperscript{106} Masetti\textsuperscript{107} et al. recently developed a novel ellipsometer based on a four detector photopolarimeter. The thickness of hydrophobic thin films formed from alkylchlorosilating agents have been accurately measured.\textsuperscript{108} The precise measurements of thickness for different length hydrocarbon chain SAMs were determined, and they were confirmed by the additional use of X-ray reflectivity by Wasserman\textsuperscript{109} et al.. It is a technique that has been widely used to measure the thickness and the refractive index of thin films for many biological applications.\textsuperscript{110} Tronin\textsuperscript{111} et al. used ellipsometry to monitor the deposition process of L-B films containing immunoglobulin IgG and to study its immunological activity. Ellipsometry
has been employed to define the refractive index of lactoperoxidase adsorbed on hydrophobic and hydrophilic silica surfaces. It was used to monitor the adsorption of IgG at methylated silica surfaces\textsuperscript{112} and at phospholipid surfaces\textsuperscript{113} to provide some information regarding adsorption modes of proteins on the surface. Moreover, competitive adsorption at hydrophobic surfaces and sequential adsorption of proteins on the surface\textsuperscript{114} were probed with the use of \textit{in situ} ellipsometry. Welin-Klintstrom\textsuperscript{115} \textit{et al.} reported that an ellipsometer equipped with a sample scanning device was capable of measuring the adsorption and desorption of proteins at liquid / solid interfaces.

A number of diffraction techniques and microscopies are worthy of mention. Surface X-ray diffraction (XRD) has been used to study the structure of ordered self-assembled monolayers. It was first used to demonstrate the well-packed structure of the thiolate monolayer when Samant\textsuperscript{116} \textit{et al.} reported the X-ray diffraction study of a docosyl mercaptan monolayer on gold (111). This study showed the SAM to be a highly ordered, hexagonal close-packed monolayer (12° tilt). Chidsey\textsuperscript{117} \textit{et al.} used the low energy helium scattering technique to provide information about the order at the upper surface of the SAM. Whitesides\textsuperscript{118} \textit{et al.} have utilized transmission electron microscopy (TEM) and electron diffraction to study the structure of docosyl mercaptan on gold (111).

Scanning tunneling microscopy (STM)\textsuperscript{119} has been used to visualize surfaces and was used to monitor the presence of holes in thiolate monolayers on gold.\textsuperscript{43,120} It could be utilized for lithography of thiolate monolayer based metalization\textsuperscript{121} and was used recently to observe and define the defect structures found for mixed monolayers of thiolate on gold.\textsuperscript{42} It could also be used for imaging biomolecules such as bovine liver catalase on the surface.\textsuperscript{122} Ultimately, an advantage of STM over the other
techniques is the possibility that STM can be used for writing and reading at a nanoscale level.\textsuperscript{123}

Atomic force microscopy (AFM)\textsuperscript{124} is a non-destructive mapping technique for surface analysis. It visualizes the spatial arrangement of monolayer assemblies on the surface and provides detailed microscopic information about monolayer structure. Alkanethiolate monolayers adsorbed on gold were investigated with the help of AFM\textsuperscript{125} as were silane coupling agents on Si wafers.\textsuperscript{126} These studies were able to clearly differentiate fluorocarbon domains vs hydrocarbon domains in the same substrate. AFM can be used to image the adsorption of protein on a surface in real time and to manipulate protein molecules adsorbed on the surface in a controlled manner.\textsuperscript{127} It was also employed to investigate the adsorption mode of surface bound proteins without significant distortion.\textsuperscript{128} and to determine how the initial spatial distribution affects the final coverage over time. It provides direct information relating to protein adsorption processes at high spatial resolution. AFM can be used to measure the adhesion forces between surfaces and the AFM probe along with the mapping of the surface.\textsuperscript{129} Also, friction measurements on the monolayer surface were performed in a recent report.\textsuperscript{130}

In addition to the techniques above, electrochemical studies\textsuperscript{131} including cyclic voltametry and impedance measurements on SAM-modified electrodes have been conducted in order to elucidate the structure and dynamic behavior of self-assembled monolayers.

1-4. Project Goal

There has been a growing interest in peptide immobilization for sensor fabrication (Figure 1-13). The specific binding of proteins to a given surface could be
utilized for constructing immunosensors with high sensitivity and specificity. The search for new methodology for attaching proteins to sensing devices in a reproducible, oriented, stable, and functional configuration brought us to design reactive chemical surfaces, which could be attached to inorganic surfaces and could bind protein molecules for immunosensor fabrication.

In the idealized sensor, a protein species is envisioned as bonded in monolayer form at the sensor surface, with its ligand binding site exposed to the bulk phase, accessible for binding the analyte, fully functional, and with little or no non-specific binding. However, it has become clear that this ideal is not attained with most current methodologies. Indeed, it seems likely that most sensors described to date probably are unevenly coated with protein, with some areas covered with protein multilayers and others with no protein at all; that some of the bound protein may be inactive; and that non-specific interactions are substantial.

![Figure 1-13. Idealized sensing surface for immunosensor fabrication.](Ab = antibody, Ag = antigen)
In order to attach proteins to inorganic surfaces, the substrate must be modified so that its surface contains a chemical functionality which is reactive with proteins. Siloxane-based self-assembled monolayers were chosen as chemical linkers for peptide attachment in organic medium. Synthetic organic chemistry is then used as a powerful tool for surface modification directed towards the construction of functionalized, organized assemblies.

A new series of SAMs, electrophilic enough to react with neutral organic sulfur compounds, were developed in this project (Chapter 2). The electrophilic SAMs allowed for peptide-attachment targeted toward HIV immunosensor construction. The relative reactivity of each electrophilic self-assembled monolayer was studied in solution as well as on the monolayer surface, and prior to peptide immobilization. The chemical attachment was monitored by a combination of surface analysis tools, primarily by the use of ESCA. The antigenic property of the peptide-bearing surface was tested, and was confirmed using ELISA and immunogold labeling (Chapter 3).
2-1. Functionalized organic surfaces

Functionalized organic surfaces (Figure 2-1, where X represents the exposed surface functionality) are of great importance in many applications which require the presence of a specific molecular functional group at a given surface. In its simplest application, SAM based on octadecyltrichlorosilane (OTS) has served as a surface modifier in some cases, e.g. to reduce the friction of the surface in micromotor application\textsuperscript{1} or to provide a hydrophobic surface for biocompatibility\textsuperscript{2}. However, it is desirable to create surfaces that can generate the basis for further modifications, for example, templates for organic SN2 reaction\textsuperscript{3} cross-linkers\textsuperscript{4} for biomolecular immobilization through covalent binding, surfaces\textsuperscript{3,5} for creation of multilayers, and templates\textsuperscript{5} for electrochemical applications.

![Functionalized organic surfaces diagram](image)

Figure 2-1. Surface modification with self-assembled monolayer. X: terminal organic functional group
Gold substrates have attracted a great deal of attention from many researchers. Alkanethiolate monolayers on gold can serve as a hydrophobic template in general, especially for phospholipid-containing bilayer formation.\textsuperscript{6} Charge-transfer groups\textsuperscript{7} built-in to a thiolate monolayer have been used for electrochemical applications. Oligoimide-bearing thiolate monolayer\textsuperscript{8} assemblies have been obtained which are similar but superior to those obtained from the L-B technique. Electro-oxidatively polymerized pyrrole-containing alkanethiolate SAMs have shown strong adhesion properties.\textsuperscript{9} Carboxylic acid-terminated thiolate monolayers\textsuperscript{10} were used for poly-L-lysine adsorption through polyionic interaction. In addition, there are porphyrin-based monolayer assemblies which have been used for electrocatalytic reduction\textsuperscript{11} and for non-linear optics.\textsuperscript{12}

Molecular recognition is a particularly interesting phenomenon (Figure 2-2). A long-chain thiolate monolayer assembly bearing a biotin functionality induced the specific adsorption of streptavidin through its four binding sites. This thiolate SAM based molecular recognition proved to be superior to that based on the L-B film, which could not be anchored to the substrate strongly enough to survive STM imaging.\textsuperscript{13} Subsequent experiments showed that these complementary templates could be used as the basis for fiber-optic\textsuperscript{14} and piezoelectric\textsuperscript{15} biosensing devices monitoring the presence of derivatized biomolecules.\textsuperscript{16}

Disulfide based glycolipid monolayers which strongly adsorbed on a gold electrode were shown to have specific binding properties for concanavalin A (Con A), as examined by cyclic voltametry.\textsuperscript{17} Carboxylate-terminated thiolate monolayers on a gold electrode provide thin films capable of selective ionic permeation and recognition.\textsuperscript{18} The thin films allowed for Cu\textsuperscript{2+} permeation and the subsequent electrochemical sensing. The recent reports regarding the cyclodextrin-immobilized
thiolate monolayer assembly\textsuperscript{19a} and calix-[4]-arene-based monolayer\textsuperscript{19b} open the door for broad application in supramolecular chemistry provided that the defects present are repaired with short chain thiolate monolayers.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{diagram.jpg}
\caption{Molecular recognition based on a thiolate SAM. The biotinylated SAM diluted with hydroxyl-bearing thiolate monolayer is exposed toward streptavidin moiety for molecular recognition. (\textsc{\smaller \textbullet}: biotin moiety; streptavidin moiety:□)}
\end{figure}
$Al_2O_3$ is also a substrate amenable to treatment with functionalized monolayer assemblies. Allara\textsuperscript{20} \textit{et al.} demonstrated the creation of a carboxyl-terminated monolayer on an alumina surface. Its structure is very similar to that of Langmuir-Blodgett films. Ogawa\textsuperscript{21} \textit{et al.} demonstrated that the carboxyl-terminated organic monolayer could be transformed into CO$_2$Me-functionalized organic surface by the action of diazomethane provided that the spatial arrangement is favorably adjusted (Figure 2-3).

![Diagram](image)

\textbf{Figure 2-3. Organic reactions on the alumina surface: Esterification}
In a different kind of *in situ* modification, a thiolate monolayer bearing *p*-nitrophenyl group on an Ag surface could be transformed into *p*-amino group by photo-induced electron transfer from the surface\textsuperscript{22} (Figure 2-4).

![Chemical structure](image)

**Figure 2-4. Organic reactions on Ag surface: Photo-induced reduction.**

Si and other hydroxylated substrates are extensively used for semiconductors and optoelectronics. A chromophoric moiety installed on the siloxane-based SAM surface has been used very effectively to demonstrate optical response\textsuperscript{23} for molecular electronics and non-linear optics. Fluorescence spectroscopy has been employed in different solvents to monitor the behavior of a pyrene-derivative constrained to the monolayer surface.\textsuperscript{24}

The use of APS (*3*-aminopropyliethoxysilane) coupling agents, to create an amine-terminated SAM, is favoured in many applications because it is relatively inexpensive and commercially available. The aminosilane-based organic monolayers have been known to serve as good templates for surface modification in
electrochemistry, and more recently for molecular patterning in microelectronics,\textsuperscript{25} surface grafting reactions,\textsuperscript{29-30} and light-directed peptide synthesis.\textsuperscript{26} This is all despite the fact that NH\textsubscript{2}-terminated SAM do not provide a well-defined structure.\textsuperscript{27} This issue has been recently discussed and it has been shown that the modified NH\textsubscript{2}-SAM surfaces are highly disordered\textsuperscript{28} regardless of surfactant chain lengths.

Kurth \textit{et al.} have reported surface reactions on thin layers of silane coupling agents which have been studied by a combination of a quartz crystal microbalance (QCM) along with XPS and ATR / FTIR (Figure 2-5). They chose the immobilized aminosilane substrates for the organic surface reaction. Pepsin was immobilized onto NH\textsubscript{2}-terminated SAM by DCC coupling through its carboxylic groups.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2-5.png}
\caption{Surface Reaction between surface-bound NH\textsubscript{2}-SAM (APS) with chlorosilane}
\end{figure}
Schick\textsuperscript{30} \textit{et al.} reported that benzenesulfonyl chloride (BSC) from the gas phase could be immobilized onto aminosilane-modified silica surface (Figure 2-6). XPS and ATR/FTIR revealed that the reaction between surface-bound primary amine and sulfonyl chloride indeed occurred, further suggesting that silane-based self-assembly methods are very useful for immobilizing specific chemical moieties onto monolayer assemblies.

![Diagram](image)

\textit{Figure 2-6. Reaction of surface-bound NH\textsubscript{2} SAM (APS) with BSC.}

However, as noted above, the use of short chain hydrophilic NH\textsubscript{2}-bearing monolayers hurts the surface packing and order (Figure 2-7). Paulson\textsuperscript{31} \textit{et al.} point out the disadvantage that resulting amide linkages are hydrolytically unstable when NH\textsubscript{2}-based SAMs are used for surface modification. They propose a general preparative route to functionalized SAMs, where preformed bromoalkylsilane SAM surfaces directly react with nucleophilic species such as pyridine. Despite the short chain (3 carbons) in the Br-terminated SAM, it still showed a greater stability compared to the hydrolytically sensitive amide linkages resulting from NH\textsubscript{2}-SAM based modification. An electrophilic SAM bearing a Cl-benzyl group, which is more electrophilic than the
corresponding alkylation could also be used for tethering a polymer via nucleophilic attack of pyridine.³

Figure 2-7. (A) Hydrolytic instability of hydrophilic and/or short chain organic monolayer assembly. (B) Hydrolytic stability of long chain organic self-assembled monolayer.
Electrophilic self-assembled monolayers were envisioned as good templates for surface modification because they are amenable to further derivatization of the functionalized surface, and can thus produce surfaces that are unobtainable from other conventional techniques. Moreover, by initially depositing a relatively hydrophobic monolayer, a high degree of order can be obtained and thus, well-packed films can undergo subsequent chemical transformation to achieve the desired ordered, functionalized surface.

\[ \text{SiX}_3 \text{SiX}_3 \text{SiX}_3 \text{SiX}_3 \quad \rightarrow \quad \text{Si}^\circ \text{Si}^\circ \text{Si}^\circ \text{Si}^\circ \quad \rightarrow \quad \text{Si}^\circ \text{Si}^\circ \text{Si}^\circ \text{Si}^\circ \]

\[ \text{SiO}_2 \quad \text{SiO}_2 \quad \text{SiO}_2 \]

**Figure 2-8.** Surface modification / transformation based on SAM. X = Br, I Y = N₃, SCN, SCOCH₃.

Balachander\(^{32}\) *et al.* previously reported successful manipulation of the composition of the surface of covalently bound siloxane-based monolayer films through the reaction of an alkyl bromide functional group with various anionic nucleophiles (Figure 2-8). It is interesting to note that in a separate study, no reaction of a Br-terminated monolayer was detected by Kurth\(^{39}\) *et al.* when using NaCN in DMF. They claimed that steric constraints were responsible for this difference.
However, the intrinsic nucleophilic and electrophilic properties may be more important in determining the chemistry occurring on the organic surface. Also, one must explicitly consider variables like solvent polarity, reaction temperature, and reaction time.

While this approach allowed for the incorporation of a variety of nitrogen and sulfur containing functional groups, the alkyl bromide SAM is insufficiently electrophilic to react with mild nucleophiles typical of neutral organic molecules. Therefore a series of electrophilic SAMs were designed as a basis for the construction of a more chemically active surfaces. Those organic surfaces, electrophilic self-assembled monolayers, were subsequently employed as substrates for peptide attachment.

![Chemical structures]

\[ X = \text{Br}(1a), \text{I}(1b) \quad X = \text{Br}(4a), \text{I}(4b) \]

\[ X = \text{Cl}(2a), \text{I}(2b) \quad X = \text{Cl}(5) \]

\[ X = \text{Br}(3a), \text{I}(3b) \quad X = \text{Br}(6) \]

Figure 2-9. The long chain alkenes and their trichlorosilane-derivatives.
In this chapter, the stable, uniform, chemically modifiable surfaces which could be obtained from silylated precursors will be described (Figure 2-9). A series of electrophilic long chain alkenes bearing halides (1a / 1b), haloacetylts (2a / 2b), and benzyl halide groups (3a / 3b) were prepared as the precursors, and the corresponding trichlorosilyl compounds (4a, 4b, 5, 6) were synthesized from them. The relative reactivities of the various electrophilic moieties in these compounds have been determined, both in solution and on surfaces. The use of these SAMs as the basis for surface modification is detailed in the last section.

2-2. Experimental Section
2-2-1. General

NMR spectra are reported in units δ and were recorded on a Varian Gemini 300 spectrometer in CDCl₃ solvent. ¹H-NMR (300 MHz) spectra are referenced to CHCl₃ at 7.24 ppm and ¹³C-NMR spectra (75 MHz) are referenced to the center of the CDCl₃ solvent triplet at 77.0 ppm. The listing of ¹³C chemical shifts includes the designation of relative peak intensity (2C, 3C, etc.) which likely indicates the number of different carbons with indistinguishable chemical shifts. Solution infrared spectra were recorded on a Perkin-Elmer 1600 Series Fourier Transform Infra-Red (FTIR) and high resolution mass spectra were recorded on a Kratos MS 25RFA spectrometer. High Performance Liquid Chromatography (HPLC) used a Waters 590 pump, a Rheodyne 7125 injector, Dynamax-60A (Si 83-121-C) semi-prep column, Dynamax-60A (C18 83-201-C) analytical column (unless otherwise indicated) and a Waters 401 Differential Refractometer. Thin Layer Chromatography (TLC) was done on aluminum backed 0.2 mm 60F254 plates from EM Science and phosphomolybdic acid was used for visualization. Column chromatography (flash) was done with silica gel
Dry tetrahydrofuran (THF) and ethyl ether were distilled from Na. Hexane for flash chromatography was distilled before use. HPLC grade hexane was used as received. HSiCl₃ (Petrarch) was distilled from quinoline. Dicyclohexyl (Aldrich, vacuum distilled) was passed through Brockman Activity I alumina (3% water by weight, 80-200 mesh, Fisher). N,N'-dimethylformamide (DMF) (Fisher) was purified by MgSO₄ drying and vacuum distillation. Acetone (Fisher) was purified by K₂CO₃ drying and distillation. Water was doubly distilled. CHCl₃ (Fisher, HPLC), p-nitrothiophenol (Aldrich), α,α'-dibromoxylene (Aldrich), and 1-decanethiol (Aldrich) were used as received. Cesium palmitate was obtained from the reaction of equimolar amount of palmitic acid and Cs₂CO₃ in water; the precipitated salt was filtered off and air dried in the hood overnight.

2-2-2. Syntheses and characterization of surfactants for self-assembled monolayers

2-2-2-01. 1-Bromo-15-hexadecene (1a)

All the surfactants used in this work were derived from commercially available undecen-1-ol (Aldrich). 1-Bromoundec-10-ene was prepared from undecen-1-ol by the action of methanesulfonyl chloride in methylene chloride at 0 °C followed by LiBr in acetonitrile for 8 h. These reactions, along with subsequent conversion to 1-bromo-15-hexadecene (1a) by a Grignard coupling reaction with 1,5-dibromopentane in well-dried THF, and 16-trichlosilyl-1-bromohexadecane (4a) by hydrosilation have been described earlier.33
2-2-2-02. 1-Iodo-15-hexadecene (1b)

1-Iodo-15-hexadecene (1b) was made from 1-bromo-15-hexadecene (1a) by the action of NaI in acetone for 4 h at RT. Compound (3.01 g, 9.96 mmol) was placed in 100 mL 1-neck round bottom flask and mixed with a solution of NaI (2.23 g, 14.88 mmol) in acetone (50 mL). The reaction mixture was stirred at RT for 4 h. The halide exchange reaction was monitored using TLC (hexane eluent). After 4 h at RT, the reaction mixture was concentrated on a rotovap, the residue was dissolved in n-hexane and precipitated NaBr was removed by filtration through a plug of silica gel. After HPLC purification (n-hexane), pure 1b was obtained: 3.37 g (97% yield).

Formula: C₁₆H₃₁I  Mass Spec. (m/e): 350.1471 (calcd), 350.1458 (found).

IR (neat): 3075, 2925, 2853, 1640, 1465, 992, 909, 720, 639.

¹H-NMR: 1.24-1.54 (m, 22H), 1.81 (m, 2H), 2.02 (m, 2H), 4.89-5.00 (m, 2H), 3.17 (t, J = 7.2 Hz, 3H), 5.80 (m, 1H).

¹³C-NMR: 7.38, 28.53, 28.94, 29.14, 29.41, 29.49, 29.53 (2C), 29.59 (3C), 30.50, 33.55, 33.81, 114.06, 139.27.

2-2-2-03. 16-Trichlorosilyl-1-iodohexadecane (4b)

Compound 1b (1.52 g, 4.34 mmol) was hydrosilated to produce 1-iodo-16-trichlorosilylhexadecane (1.65 g, 3.40 mmol, 78% yield) by reaction of HSiCl₃ in the presence of Pt catalyst in a pressure tube for 8 hr at RT. Pure compound 4b was obtained from kugelrhor distillation (115-125°C under 5 mmHg). It was stored in a desiccator.

IR (neat): 2926, 2853, 1465, 765, 721, 691, 639.

¹H-NMR: 1.24-1.53 (m, 28H), 1.80 (m, 2H), 3.17 (t, J = 6.9 Hz, 2H).
\(^{13}\)C-NMR: 7.38, 22.25, 24.83, 28.54, 29.09, 29.35, 29.43, 29.55, 29.64, 29.82 (2C), 29.86, 30.52, 31.81, 33.57, 33.60.

2-2-2-04. 1-Acetoxy-15-hexadecene (7)

Into a dry, three neck 500 mL flask fitted with a magnetic stirring bar was placed potassium acetate (6.42 g, 65.4 mmol) and DMF (150 mL). Compound 1a (16.42 g, 54.3 mmol) was added at RT and the reaction mixture was heated and maintained at 80°C. The reaction progress was monitored by noting the disappearance of compound 1a by TLC (n-hexane). After completion, the reaction mixture was cooled to room temperature. H\(_2\)O (150 mL) was added, and the product was extracted with two portions of diethyl ether (2 x 300 mL). The combined ether extracts were dried over anhydrous MgSO\(_4\) and concentrated on a rotovap. The crude product was purified by flash chromatography (silica gel / hexane): The yield was 14.26 g (93%).

IR (neat): 3076, 2925, 2854, 1744, 1641, 1466, 1365, 1040, 993, 909, 722.

\(^1\)H-NMR: 1.23-1.28 (m, 22H), 1.59 (m, 5H), 2.00 (m, 2H), 4.03 (t, \(J = 6.6\) Hz, 2H), 4.92 (m, 2H), 5.78 (m, 1H).

\(^{13}\)C-NMR: 21.03, 25.89, 28.57, 28.93, 29.14, 29.50, 29.56 (3C), 29.64 (4C), 33.81, 64.68, 114.06, 139.27, 169.82.

2-2-2-05. 15-Hexadecen-1-ol (8)

Compound 7 (4.30 g, 15.2 mmol) was placed in 250 mL one-neck round bottom flask containing 100 mL of solvent (70 % ethanol : 30 % water). KOH (1.28 g, 22.8 mmol) in 150 mL of ethanol : water (70 : 30) was added to the reaction vessel. The reaction mixture was heated to reflux for 2 h. The resulting mixture was cooled to
RT and was concentrated on a rotovap to evaporate the ethanol. The product was extracted with diethyl ether (2 x 150 mL), dried over MgSO₄, filtered through a Buchner funnel, and concentrated on a rotovap. It was initially purified by flash chromatography (hexane / silica) and ultimately by normal phase HPLC (n-phase: 20% EA ; 80% n-hexane). The yield was 3.25 g (89 %).

IR (CCl₄): 3695, 3628, 3078, 2981, 2927, 1166, 995, 912, 722.

¹H-NMR: 1.24-1.35 (m, 22H), 1.54 (m, 2H), 2.03 (m, 2H), 3.62 (t, J = 6.6 Hz, 2H), 4.94 (m, 2H), 5.78 (m, 1H).

¹³C-NMR: 26.14, 29.36, 29.56, 29.84 (3C), 29.92 (4C), 30.06, 33.22, 34.23, 63.52, 114.48, 139.69.

2-2-2-06. 1-Chloroacetoxy-15-hexadecene (2a)

15-Hexadecen-1-ol (5.4 g, 22.46 mmol) was placed in a 250 mL three neck round bottomed flask with methylene chloride (100 mL) and pyridine (1.79 g, 22.63 mmol) at RT and was cooled to -4 °C. Chloroacetyl chloride (2.79 g, 24.6 mmol) was placed in a dropping funnel and was slowly added to the reaction mixture over 40 minutes at -4 °C. The temperature was raised to RT after 2 h of stirring at -4 °C. The salt formed in the process was removed by passing the reaction mixture through a small pipette filled with anhydrous MgSO₄ and the solution was concentrated on a rotovap. It was purified by flash chromatography and HPLC (20% EA : 80% n-hexane). The yield was 6.39 g (90 %)

Formula: C₁₈H₃₃ClO₂ Mass Spec. (m/e): 316.2169 (calcd), 316.9987 (found).
IR (CCl₄): 3695, 3628, 3078, 2981, 2927, 1166, 995, 912, 722.

¹H-NMR: 1.24-1.35 (m, 22H), 1.54 (m, 2H), 2.03 (m, 2H), 3.62 (t, J = 6.6 Hz, 2H), 4.94 (m, 2H), 5.78 (m, 1H).
13C-NMR: 26.14, 29.36, 29.56, 29.84 (3C), 29.92 (4C), 30.06, 33.22, 34.23, 63.52, 114.48, 139.69.

2-2-2-07. 1-Chloroacetoxy-16-(trichlorosilyl)hexadecane (5)

1-Chloroacetoxy-15-hexadecene (1.02 g, 3.23 mmol) was hydrosilylated using 3 mL of HSiCl3 in the presence of chloroplatinic acid at RT for 4 h to give 1-chloroacetoxy-16-trichlorosilylhexadecane 5. The yield was 0.95 g (65%) after kugelrhoro purification at 117-128 °C under 5 mm Hg.

IR (neat): 3436, 2926, 2854, 1762, 1741, 1466, 1413.

1H-NMR: 1.24-1.41 (m, 30H), 4.04 (s, 2H), 4.16 (t, J = 6.6 Hz, 2H).


2-2-2-08. 1-Iodoacetoxy-15-hexadecene (2b)

1-Chloroacetoxy-15-hexadecene (6.02 g, 19.04 mmol) was placed in a 250 mL one-neck round bottomed flask at RT. NaI (4.28 g, 28.55 mmol) was dissolved in acetone (150 mL), and this solution was added to the reaction flask and stirred for 2 h. Acetone was evaporated after the reaction was complete, n-hexane (45 mL) was added to the mixture. The reaction mixture was filtered through a small amount of silica gel to remove the residual NaI and the NaBr. It was concentrated on a rotovap and purified by flash chromatography. (silica gel / 10% ethyl acetate : 90% n-hexane) The final purification was performed by HPLC (10% EA : 90% n-hexane). The yield was 7.30 g (94%).
MS (m/e): 408.1525 (calcd) C_{17}H_{31}O_2 (M^+-I); 281.2428 (found), 281.2480 (calcd), C_{16}H_{31}O (M^+-COCH_3); 239.2248 (found), 239.2375 (calcd), C_{15}H_{31} (M^+-OCOCH_3); 223.2446 (found), 223.2425 (calcd).

IR (neat): 3075, 2923, 2852, 1734, 1640, 1465, 1415, 1137, 993, 909, 721, 650.

H-NMR: 1.24-1.33 (m, 22H), 1.62 (m, 2H), 2.01 (m, 2H), 3.66 (s, 2H), 4.11 (t, J = 6.9 Hz, 2H), 4.94 (m, 2H), 5.79 (m, 1H).

C-NMR: -5.33, 25.75, 28.35, 28.38, 28.95, 29.15, 29.22, 29.49, 29.62 (2C), 29.65 (3C), 33.82, 66.29, 114.08, 139.27, 168.27.

2-2-2-09. 1-p-Bromotolyl-11-dodecene (3a)

A flame-dried three neck 250 mL flask equipped with a pressure-equalizing addition funnel, a reflux condenser with a N_2 inlet, and a magnetic stirring bar was charged with Mg turnings (4 g, 164.54 mmol). The addition funnel was charged with a solution of o-undecenyl bromide (15.60 g, 66.90 mmol) in 100 mL of dry THF. A portion of this solution (30 mL) was added to the flask, and it was gently warmed to initiate the Grignard coupling reaction. Once Grignard formation began, the rest of the solution was added over a period of 30 min. After the contents of the flask were heated at reflux for an additional 1 h, it was cooled to RT. To another three-neck 500 mL flame dried flask, fitted with a pressure equalizing addition funnel, magnetic stirring bar, and α,α'-dibromoxyylene (21.20 g, 80.32 mmol). This solution was cooled in an ice / salt bath to -10 °C. The Grignard reagent from the first flask was transferred to the addition funnel by syringe. It was then added to the reaction flask. A 0.2 M solution of LiCl and CuCl_2 in THF (1.5 mL) was added to the reaction flask. The resulting mixture was stirred at -10 °C for 12 h. Ethyl ether (200 mL) was added to the flask, and the entire contents were transferred to a separatory funnel. The organic
solution was washed twice with saturated aq. NH₄Cl solution. and once with saturated aqueous NaCl solution, dried over anhydrous MgSO₄, and concentrated on a rotovap. The excess dibromoxylene was removed by flash chromatography (n-hexane) and the reaction product was purified by HPLC (Whatman Magnum 20 column, n-hexane, 20 mL/min); The yield was 8.33 g (37%).

Formula: C₁₉H₂₉Br
MS m/e: 336.1453 (calcd), 336.1423 (found).
IR (neat): 2921, 2852, 3081, 3054, 1643, 1615, 1463, 1437, 991, 964, 837, 760, 724.
¹H-NMR: 1.25-1.35 (m, 14H), 1.55 (m, 2H), 2.03 (m, 2H), 2.57 (t, J = 7.5 Hz, 2H), 4.48 (s, 2H), 4.99 (m, 2H), 5.79 (m, 1H), 7.13 (d, J = 8.0 Hz, 2H), 7.29 (d, J = 8.0 Hz, 2H).
¹³C-NMR: 28.94, 29.14, 29.48, 29.54, 29.58 (2C), 31.35, 33.81, 35.69, 114.09, 128.84 (2C), 128.96 (2C), 134.97 (2C), 139.25, 143.44.

2-2-2-10. 1-p-(Bromotolyl)-12-(trichlorosilyl)dodecane (6)

1-p-Bromotolyl-12-dodecene (1.20 g, 3.57 mmol) was hydrosilylated with HSiCl₃ with added chloroplatinic acid catalyst at RT for 14 hrs. It was purified by kugelrhor distillation. The yield was 50% (0.86 g, 1.95 mmol).
IR (neat): 3010, 2930, 2855, 1614, 1466, 832, 757, 722, 692.
¹H-NMR: 1.24-1.41 (m, 20H), 1.60 (m, 2H), 2.57 (t, J = 7.5 Hz, 2H), 4.48 (m, 2H), 7.13 (d, J = 8.0 Hz, 2H), 7.28 (d, J = 8.0 Hz, 2H).
¹³C-NMR: 22.24, 24.31, 26.83, 29.00, 29.14, 29.30, 29.48, 29.55, 31.35, 31.59, 31.80, 33.81, 35.69, 128.84 (2C), 128.96 (2C), 134.97, 139.25.
2-2-11. 1-\textit{p}-Iodotolyl-12-dodecene (3b)

\textit{p}-Bromotolyl-12-dodecene (2.53 g, 7.53 mmol) was placed in a 100 mL one-neck round bottomed flask with NaI (1.69 g, 11.27 mmol) in acetone (50 mL). It was stirred for 2 h and the reaction went to completion (TLC monitoring in \textit{n}-hexane). Acetone was removed by distillation and \textit{n}-hexane (30 mL) was added to the flask. The resultant mixture was filtered through a thin layer of silica gel and was concentrated on a rotovap. It was purified by flash chromatography and finally by HPLC (\textit{n}-hexane). The yield was 2.72 g (94%).

MS m/e: C_{19}H_{29} (M^+I); 257.2324 (found), 257.2269 (calcd) (M^+-CH_{2}PhCH_{2}I); 153.1494 (found), 153.1643 (calcd).

IR (neat): 3078, 2928, 2855, 1641, 1613, 1466, 993, 912, 842, 760, 720.

\textsuperscript{1}H-NMR: 1.24-1.37 (m, 14H), 1.54 (m, 2H), 2.03 (m, 2H), 2.54 (t, J = 7.5 Hz, 2H), 4.44 (s, 2H), 4.93 (m, 2H), 5.78 (m, 1H), 7.08 (d, J = 8.1 Hz, 2H), 7.27 (s, J = 8.1 Hz, 2H).

\textsuperscript{13}C-NMR: 6.24, 28.90, 28.93, 29.12, 29.36 (2C), 29.56, 29.62, 31.29, 33.81, 35.68, 114.08, 128.61 (2C), 128.86 (2C), 136.37, 139.25, 142.88.

2-2-3. Syntheses and characterization of products from solution kinetics

The following materials were prepared and characterized separately for use as HPLC standards. Reactions between \textit{p}-nitrothiophenol (PNTP) and 1b, 2b, and 3b to make 1c, 2c, and 3c respectively, were each done by stirring solutions of the reactants in DMF, under N\textsubscript{2} at room temperature in the presence of triethylamine. The products were separated by flash chromatography (30% EA: 70\% \textit{n}-hexane) followed by HPLC (acetonitrile, reverse phase, semi-prep column, flow rate: 20 mL / min, chart rate: 30 cm / h).
2-2-3-1. 1-\(p\)-Nitrothiophenyl-15-hexadecene (1c)

IR (CCl₄): 3077, 2929, 2856, 1641, 1520, 1465, 1340, 995, 914.

\(^1\)H-NMR: 1.24-1.43 (m, 22H), 1.65-1.75 (m, 2H), 2.00 (m, 2H), 2.99 (t, 2H, J = 7.5 Hz), 4.92 (m, 2H), 5.78 (m, 1H), 7.27 (d, J = 9 Hz, 2H), 8.09 (d, J = 9 Hz, 2H).

\(^{13}\)C-NMR: 28.46, 28.86, 28.92, 29.11 (2C), 29.44 (3C), 29.61 (4C), 31.93, 33.80, 114.06, 123.90 (2C), 125.95 (2C), 139.23, 148.28.

2-2-3-2. 1-(\(p\)-Nitrothiophenyl)acetoxy-15-hexadecene (2c)


\(^1\)H-NMR: 1.22-1.37 (m, 22H), 1.59 (m, 2H), 2.03 (m, 2H), 3.75 (s, 2H), 4.12 (t, 2H, J = 6.6 Hz), 4.92 (m, 2H), 5.78 (m, 1H), 7.41 (d, 2H, J = 9Hz), 8.13 (d, 2H, J = 9 Hz).

\(^{13}\)C-NMR: 25.76, 28.45, 28.69, 28.79, 28.93, 29.14 (2C), 29.49 (4C), 29.62, 33.81, 34.57, 66.27, 114.06, 124.04 (2C), 126.74 (2C), 139.25, 145.49 (2C), 168.67.

2-2-3-3. 1-(\(p\)-Nitrothiophenyl)benzyl-11-dodecene (3c)


\(^1\)H-NMR: 1.24-1.37 (m, 14H), 1.56 (m, 2H), 2.00 (m, 2H), 2.56 (t, 2H, J = 7.5 Hz), 4.20 (s, 2H), 4.93 (m, 2H), 5.79 (m, 1H), 7.12 (d, J = 9 Hz, 2H), 7.31 (m, 4H), 8.08 (d, J = 9 Hz, 2H).
\[ ^{13}\text{C-NMR:} \ 28.90, \ 29.09, \ 29.26, \ 29.43, \ 29.53, \ 29.57, \ 31.35, \ 33.76, \ 35.56, \ 36.73, \ 114.04, \ 123.86 \ (2\text{C}), \ 126.44 \ (2\text{C}), \ 128.55 \ (3\text{C}), \ 128.82 \ (2\text{C}), \ 132.38 \ (2\text{C}), \ 139.21, \ 142.65, \ 147.37. \]

2-2-4. Surface modification with electrophilic self-assembled monolayers

2-2-4-1. Preparation of solid substrates for monolayer coating

The glass slides used in this work were obtained from Dynalab Corp. (10 x 10) and were cleaned by washing with doubly-distilled water followed by cleaning with hot CHCl₃ in a Soxhlet extractor for 30 min. The substrates were then dried. The Ge and ZnSe ATR crystals were cleaned with hot CHCl₃ for 30 min in a Soxhlet extractor. All the substrates were plasma-cleaned for about 30 min in a radio-frequency Argon plasma (Harrick PDC-3xG Plasma Cleaner), stored in fluorocarbon containers (Fluoroware Inc.), and used within 12 h.

2-2-4-2. Preparation and use of coating solutions

All trichlorosilyl surfactants made in this work were used as $5 \times 10^{-3}$ M solutions in dicyclohexyl. The surfactants (100 \muL) were added to the dicyclohexyl solvent (5 mL) under inert atmosphere and transferred to the bench top. All the surfactant solutions were used by immersion of the substrate (using Teflon-coated tweezers) into a 10-mL beaker containing about 5 mL of the surfactant solution and a magnetic stirrer. The substrate is quickly withdrawn from the solution after 30 min, washed with CHCl₃ and water, and finally cleaned in hot CHCl₃ in a Soxhlet extractor for 10 min.
2-2-4-3. Contact angle measurements

Contact angles were measured by using a Rame-Hart Model 100 contact angle goniometer equipped with a controlled environment chamber. Advancing contact angles were determined by placing a drop of H₂O from a syringe, advancing the periphery of the drop by adding more liquid, withdrawing the syringe, and measuring the advancing contact angle within 30 sec of application of the drop. Receding contact angles were measured by withdrawing part of the liquid from the top and measuring the angle. The temperature of the measurements was not controlled but stayed within a range of 22 ± 2 °C. Reported values are averages of four to six measurements taken at different points on the surface.

2-2-4-4. X-ray photoelectron spectroscopy (XPS)

ESCA measurements were carried out on a Perkin-Elmer ESCA 5400 instrument. Analyses were done by using Mg Kα lines at a pressure of 10⁻⁹ Torr with a take-off angle of 45 °. Survey spectra were recorded on a 1-mm spot, using 150 eV pass energy, 200 W electron beam power, and an acquisition time of 30 min. Peak positions were assigned by referencing the C 1s peak at 284.7 eV.

2-2-4-5. In situ transformations of monolayer functionality

Transformations were carried out by dipping the monolayer-coated substrates (glass slides or ATR crystal) into the reagent solution under the indicated reaction conditions for the indicated time and then rapidly withdrawing it with teflon-coated tweezers. Unless stated otherwise, all monolayer-coated substrates were cleaned after they were withdrawn from the reaction medium by washing with acetone and
methylene chloride. before finally cleaning with hot methylene chloride in a Soxhlet extractor. Characterization of the modified films involved contact angle measurements, IR, and XPS.

2-2-4-6. In situ reaction of Br (Cl) to I as a halide exchange

In a dry 100 mL Erlenmeyer flask containing a small teflon-coated magnetic bar were placed NaI (0.3 g) and 20 mL of dry acetone (0.1 M). The monolayer-coated substrates were placed in the flask, and the mixture was stirred at room temperature for 4 h. The substrates were removed from the flask and cleaned with dry acetone and subsequently methylene chloride.

2-2-4-7. Solution kinetics of surfactant precursors

Stock solutions (0.1 M) of compounds 1b, 2b, 3b and p-nitrothiophenol (PNTP) were prepared in DMF that had been dried over anhydrous MgSO₄. After 100 μL of each of the above electrophiles was placed in each of 10 small vials with a magnetic stirring bar, 150 μL of p-nitrothiophenol (1.5 eq.) was added to each reaction vessel at RT, and the reactions were monitored by injecting 100 μL of reaction mixture from each vessel into the HPLC column at specific time intervals. In another set of experiments, 100 μL of compounds 1a and 1b were each placed into separate vials with a magnetic stirring bar, 150 μL of PNTP was added to each reaction vial, and the mixtures were stirred for times up to 72 h. Aliquots (100 μL) of these reaction mixtures were taken from the vial and injected into the HPLC column. The relative reactivity of 1a-1b was assessed by the amount of product as measured by HPLC. Each set of 2a-3a, 3a-3b, and 2a-2b was used for assessing relative reactivities in a similar manner. (Eluent: acetonitrile; chart rate: 30 cm / h; reverse
phase analytical column used). Samples were injected into the HPLC column after a given time, and the disappearance of starting materials, and appearance of products (1c, 2c, and 3c) were monitored. At an HPLC flow rate of 0.5 mL/min, retention times (min) were: (23.6); (27); (18.4); (23). At a flow rate of 1 mL/min, retention times (min) were: (27.2); (19.4).

2-3. Results and Discussion

A series of electrophilic SAMs were designed as a basis for the construction of chemically active SAM surfaces. They were subsequently employed as reactive points of attachment for neutral organic (primarily thiol) nucleophiles.

2-3-1. Preparation of the electrophilic alkenes and their trichlorosilanes

We synthesized and studied the chemistry of a series of electrophilic long chain alkenes bearing a variety of functionality (Figure 2-9). The syntheses of these compounds were all straightforward (Figure 2-10). Compound is the precursor to (NaI/acetone) and to 2a (conversion of the Br to OH via the acetate, followed by esterification with chloroacetyl chloride). Compound 2b is derived from (NaI/acetone). Compound 3a is made by the Grignard coupling of 1-bromo-10-undecene with α,α'-dibromo-p-xylene, and is obtained by halogen exchange. Hydrosilylation of each of these olefins gave rise to the respective alkyltrichlorosilanes (4-6). Due to the relative instability of iodide-bearing versions of 5 and 6, insufficient amounts for proper characterization and subsequent use were obtained.
Figure 2-10. The synthetic routes to the trichlorosilanes.

2-3-2. Preparation and characterization of electrophilic SAMs

Compounds 4-6 were deposited from dilute dicyclohexyl solution as covalently bound, siloxane-anchored monolayer films. Iodide bearing SAM surfaces were created by direct deposition (4b) or by exchanging bromide or chloride for iodide on an already deposited film. The wetting properties of each SAM were determined. All directly deposited films showed contact angle hysteresis of 3-4 degrees, and the films created by in situ halide exchange (α-iodoacetyl and benzyl iodide) showed hysteresis of 5 and 6 degrees, respectively. Water contact angles and distinctive ESCA features
of the monolayers on glass are listed in Table 2-1. The advancing contact angles for each SAM provided a measure of their relative hydrophobicities. XPS analysis of each surface showed the expected carbon polymethylene peak (284.7 eV) along with a carbon signal at 293.8-293.9 eV for the halogen-bearing carbon and a peak at 289.0-289.1 eV for the carbonyl of the haloacetate surfaces. Halogen XPS peaks for Cl (199 eV), Br (70-70.5 eV), and I (618.9-620.6 eV) were easily detected.

<table>
<thead>
<tr>
<th>FILM (source)</th>
<th>Advancing Contact Angle (°)</th>
<th>Receding Contact Angle (°)</th>
<th>Halogen Signal (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>alkyl Br</td>
<td>84</td>
<td>80</td>
<td>70.5 (Br)</td>
</tr>
<tr>
<td>alkyl I</td>
<td>85</td>
<td>82</td>
<td>620.3 (I)</td>
</tr>
<tr>
<td>in-situ alkyl I</td>
<td>NaI, acetone</td>
<td>85</td>
<td>620.3 (I)</td>
</tr>
<tr>
<td>Cl-acetyl</td>
<td>69</td>
<td>65</td>
<td>199.0 (Cl)</td>
</tr>
<tr>
<td>in-situ I-acetyl</td>
<td>NaI, acetone</td>
<td>71</td>
<td>618.9 (I)</td>
</tr>
<tr>
<td>benzyl Br</td>
<td>86</td>
<td>82</td>
<td>70.0 (Br)</td>
</tr>
<tr>
<td>in-situ benzyl I</td>
<td>NaI, acetone</td>
<td>88</td>
<td>620.6 (I)</td>
</tr>
</tbody>
</table>

Table 2-1. Water contact angles and distinctive XPS of monolayers on glass.

In general, surfaces modified with electrophilic SAMs were relatively hydrophobic. The benzyl iodide and α-iodoacetyl SAMs were found to be quite labile. To retain an iodide-bearing SAM, precautions with regard to trace water in solvents, atmospheric moisture, and light were required, and generation of the α-hydroxyl derivative of the ester group (presumably by trace moisture) was apparent by NMR in
some experiments. Thus, direct SAM formation with the iodide form of these compounds was not undertaken. Rather, the more stable bromo- and chloro-compounds were deposited and iodide was exchanged onto the surface as needed. The halide exchange is very useful in activation of the organic surface for further transformation.

2-3-3. Preparation of iodide-bearing SAMs derivatized from halide exchange

The halide exchange reaction, known as the Finkelstein reaction,\textsuperscript{34} is a typical $S_N2$ reaction. It takes advantages of the superior solubility in acetone of NaI over NaBr and NaCl as a driving force for completion. It can be used for producing the favorably reactive iodide derivative for chemical transformations on the surface (eq. 2-1).

$$\text{RT or Reflux}$$

$$\text{R-Cl (Br) SAM} \xrightarrow{\text{NaI / acetone}} \text{R-I SAM} \quad \text{(eq. 2-1)}$$

The XPS experiments in Figure 2-10 illustrate a monitoring of the displacement chemistry by XPS. I-bearing SAMs were prepared from corresponding Br / Cl containing compounds. Initially-deposited Br-alkyl SAM (glass / Si wafer) gave rise to the Br peak seen in the ESCA. They were converted into I-alkyl SAM by the action of NaI within 4 h at RT, as evidenced by the appearance of a strong I peak and the disappearance of the initial Br peak (Figure 2-11 A). Complete reaction could be also achieved within 1 h in refluxing acetone (Figure 2-11 B). The halide exchange reaction on the surface went to completion within 1 h at RT for Cl-acetyl SAM (Figure 2-11 C).
Figure 2-11 A. Halide exchange reaction of Br-alkyl SAM. (a) Br region of initially-deposited Br-alkyl SAM. (b) Br region of I-alkyl SAM derivatized from Br-alkyl SAM by reaction with NaI in acetone at RT for 4 h. (c) I region of initially-deposited Br-alkyl SAM. (d) I region of I-alkyl SAM derivatized from Br-alkyl SAM by reaction with NaI in acetone at RT for 4 h. I-alkyl SAM could be achieved by reaction of Br-alkyl SAM with NaI. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
Figure 2-11 B. Comparison of two routes to the same I-alkyl SAM. (a) Br region of in-situ modified I-alkyl SAM for 1 h in refluxing acetone. (b) I region of I-alkyl SAM from derivatization. (c) I region of I-alkyl SAMs from initial deposition. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
Figure 2-11 C. I-acetyl SAM derivatized from Cl-acetyl SAM. (a) Cl region of Cl-acetyl SAM; (b) Cl region of in situ modified I-acetyl SAM; (c) I region of in situ modified I-acetyl SAM. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
X-ray beam damage to Br / I-terminated SAMs has been reported.\textsuperscript{38} Nevertheless, repeated XPS measurements under standard conditions permitted a determination of the relative stability of our functionalized films and the reproducibility of these measurements. It was clear that our films were sufficiently stable for meaningful XPS analysis of the SAM surface. Although the iodide signal was somewhat variable in intensity, multiple experiments of this sort confirmed that the expected exchange reaction occurs; i.e. an iodide signal always developed and little or no signal from bromide or chloride remained. These results suggested that the desired iodoacetyl functionality is successfully installed on the surface; however, these experiments do not establish that an uniform layer of iodide is present.

2-3-4. Functionalization of electrophilic SAMs

The functionalized organic surfaces, prepared by the above strategy, were used for further surface modifications. Among the six electrophilic SAM surfaces tested, BzBr / BzI / I-acetyl SAMs show promise for immobilizing even mildly nucleophilic compounds by nucleophilic displacement reactions. I-Acetyl SAM was the most reactive and was shown to react with even the very unreactive nucleophile octadecanethiol (ODT) under conditions where other functionalized SAM surfaces did not react. This was shown by following S and Br signals of reacted SAM surfaces (Figure 2-12). The reaction scheme depicted therein (conversion of chloroacetyl to iodoacetyl, followed by reaction with octadecanethiol) was followed by the disappearance of each of the halide signal and by the ultimate incorporation of the thiol as evidenced by the S XPS signal.
Figure 2-12. ESCA of I-acetyl SAM treated with ODT: X = Cl, Y = I, and R = octadecyl. The scheme represents Cl-acetyl SAM (upper-left), I-acetyl SAM (upper-right) wherein initial SAM attachment of Cl-acetyl functionality is followed by exchange of Cl for I and subsequent reaction with an alkanethiol (octadecanethiol). (a) S signal (bottom-left) and (b) I signal (bottom-right) in the ESCA. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
Figure 2-13. The attachment of palmitic acid salt onto I-acetyl SAM. XPS signals of Cl region (a) and I region (b) for the SAM surface modified with palmitic acid salt. The scheme (upper) represents how the SAM surface is obtained. The Y axis is relative signal intensity in counts per electronvolts with the largest signal (peak or noise) normalized to a value of 10 units.
The usefulness of the strongly electrophilic I-acetyl SAM was also shown by its reaction with an alkylcarboxylate (Figure 2-13). Such nucleophiles are non-reactive toward Br-alkyl or I-alkyl SAM under identical conditions (not shown in Figure 2-13). The cesium salt of palmitic acid, made from palmitic acid and Cs₂CO₃, reacts with I-acetyl SAM. The drastic change in wetting property (from water adv. CA: 68° to water adv. CA: 104°) was the primary evidence that the reaction occurred. The disappearance of the I peak in the ESCA further supported this.

2-3-5. The effect of solvent used for SAM transformation

The effect of solvent on SAM transformations was explored with the goal of maximizing surface reaction. The reaction of a Br-alkyl SAM with NaI, when done in acetonitrile did not go to completion within 12 h, whereas this reaction is complete in acetone within 4 h (Figure 2-14). The residual Br peak ratio relative to the I peak (38 %) clearly shows the incomplete conversion.

<table>
<thead>
<tr>
<th>Element</th>
<th>Area (cts-eV/s)</th>
<th>Sensitivity Factor</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br 3d</td>
<td>2337</td>
<td>1.053</td>
<td>62.29</td>
</tr>
<tr>
<td>I 3d</td>
<td>8538</td>
<td>6.206</td>
<td>37.71</td>
</tr>
</tbody>
</table>

Figure 2-14. Br-alkyl SAM treated with NaI in acetonitrile at RT for 12 h. XPS signals of Br (a) and I (b) peaks of the reacted Br-alkyl SAM in acetonitrile. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
The most complete reaction of nucleophilic species with the I-acetyl SAM surface was obtained from reaction in DMF. In many cases, the same reaction did not occur when the solvent system was changed from DMF to methylene chloride or acetone. The reaction of I-acetyl SAM with p-nitrothiophenol (PNTP) in methylene chloride did not give any indication of product formation upon prolonged exposure to the reagent solution, whereas DMF provided the medium for complete conversion. It must be noted that some solvents damage the SAM. DMSO at 150 °C for a few h removed the monolayer of OTS from the glass slides, and thus could not be compatible with any monolayer transformations.

2-3-6. Reactivity of various electrophiles : solution chemistry

In order to assess the utility of each of the SAMs as electrophilic attachment vehicles, the relative electrophilicity of the various functionalities, as determined from their reactivity with organic thiols, was studied in solution. All reactions were run in dry distilled DMF at room temperature. With the focus on relative reactivity no attempt was made to precisely determine rate constants. Experiments were done wherein pairs of electrophilic substrates (1a, 1b, 2a, 2b, 3a, 3b) were reacted in competition with each other for a limited amount of thiol (PNTP - more reactive; or DT - less reactive). These were in addition to a systematic set of parallel experiments where each electrophile was reacted with one of the thiol nucleophiles and the formation of product was monitored as a function of time. An illustrative set of such data, obtained for compounds 1b, 2b, and 3b, reacted with PNTP, is shown in Table 2-2. It clearly demonstrates that under conditions where the half life of the iodoacetyl was less than 3 minutes, the benzyl iodide had a half life of 10-15 minutes, and the alkyl iodide had a half life of approximately one day. This data set provides a
particularly valuable basis for comparison to the SAMs bearing these same three functionalities (discussed in section 2-4-7 and summarized in Figure 2-17).

<table>
<thead>
<tr>
<th>Electrophile</th>
<th>1b</th>
<th>2b</th>
<th>3b</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 min</td>
<td>2</td>
<td>32</td>
<td>66</td>
</tr>
<tr>
<td>10 min</td>
<td>4</td>
<td>49</td>
<td>84</td>
</tr>
<tr>
<td>15 min</td>
<td>6</td>
<td>50</td>
<td>84</td>
</tr>
<tr>
<td>20 min</td>
<td>6</td>
<td>55</td>
<td>85</td>
</tr>
<tr>
<td>25 min</td>
<td>8</td>
<td>56</td>
<td>87</td>
</tr>
<tr>
<td>30 min</td>
<td>11</td>
<td>66</td>
<td>97</td>
</tr>
<tr>
<td>24 h</td>
<td>54</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>48 h</td>
<td>78</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>72 h</td>
<td>89</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2-2. Product formation (%) of I-bearing electrophiles toward PNTP.

2-3-7. Relative reactivity of SAMs: surface chemistry

A number of systems where thiol displacement of halide leaving groups led to a single uniform well-characterized SAM can be described. The structures of these films are shown in Figure 2-16.

The wetting properties of each SAM were demonstrated to show hysteresis of 4~5 degrees. Water contact angles and distinctive ESCA features of the monolayers on glass are listed in Table 2-2.

Having established the relative electrophilicities of the various functional groups in solution, and having demonstrated that reaction of the SAM can produce newly functionalized SH-bearing surfaces, we turned to the relative reactivity of the electrophilic SAMs. The reactivity of these SAMs with PNTP and DT was examined
Figure 2-15. The structures of the SAM films. (A): I-alkyl SAM treated with PNTP (R=p-nitrophenyl); (B-1): PNTP treated I-acetyl SAM (R=p-nitrophenyl); (B-2): decanethiol-treated I-acetyl SAM (C): PNTP-treated Bzl SAM (R=p-nitrophenyl).

<table>
<thead>
<tr>
<th>SAM</th>
<th>Preparation</th>
<th>ESCA (eV)</th>
<th>Contact Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM A</td>
<td>I-alkyl SAM treated with PNTP in DMF</td>
<td>284.7, 294.0 (C1s)</td>
<td>81+/−1 (adv.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>164.0 (S2p), 398.9 (N1s)</td>
<td>75+/−2 (rec.)</td>
</tr>
<tr>
<td>SAM B-1</td>
<td>I-acetyl SAM treated with PNTP in DMF</td>
<td>284.7, 289.0, 293.6 (C1s)</td>
<td>75+/−1 (adv.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>164.0 (S2p), 399.8 (N1s)</td>
<td>70+/−2 (rec.)</td>
</tr>
<tr>
<td>SAM C</td>
<td>BzBr SAM treated with PNTP in DMF</td>
<td>284.7, 293.7 (C1s)</td>
<td>78+/−1 (adv.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>163.8 (S2p), 399.0 (N1s)</td>
<td>75+/−2 (rec.)</td>
</tr>
<tr>
<td>SAM B-2</td>
<td>I-acetyl SAM treated with decanethiol (DT) in DMF</td>
<td>284.7, 289.0 (C1s)</td>
<td>72+/−2 (adv.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>163.9 (S2p)</td>
<td>68+/−2 (rec.)</td>
</tr>
</tbody>
</table>

Table 2-3. The ESCA and wetting properties of modified surfaces.
Figure 2-16. Reactivity of I-bearing electrophilic SAMs on the surface against PNTP. XPS monitoring of p-nitrothiophenol reaction with three different kinds of SAMs: (top) nitrogen signal for alkyl iodide (1), benzyl iodide (2), and iodoacetyl (3) SAMs treated with the thiol as described in the experimental section; (middle) iodide signal retained on SAMs formed with alkyl iodide (3), iodoacetyl (2), and benzyl iodide (1) compounds; (bottom) sulfur signal seen with iodoacetyl (1), benzyl iodide (2), and alkyl iodide (3) SAMs following treatment with the thiol. The Y axis is offset and only shows relative intensity.
by XPS. Figure 2-16 shows the relative reactivity of three I-bearing SAMs against p-nitrothiophenol (PNTP). Following three hours treatment, both the benzyl iodide and the α-iodoacetyl surfaces showed clear nitrogen and sulfur signals, with no remaining iodide signal. The sulfur and nitrogen signals were stable to extensive washing with organic solvents and water, and to refluxing overnight in methylene chloride. Thus, the thiol is strongly attached to the SAM surface, and the data strongly supports the notion that nucleophilic displacement of iodide by thiol has occurred.

Both PNTP and DT reacted readily, but since PNTP was much more reactive DT and since its attachment to the surfaces could be followed by examination of both sulfur and nitrogen (from its nitro group) signals, it was used as the basis for comparing the surfaces. The reactions of six different electrophilic SAMs with PNTP are presented in Fig. 2-17. The relative reactivity was evaluated simply by monitoring the appearance of N / S signals and disappearance of I signal before and after reaction. Thus, the relative reactivity of each SAM was established by ESCA analysis of the product surface.

2-4. Concluding remarks

The use of organic monolayer assemblies for surface modification allowed us to immobilize organic molecules by covalent binding with no noticeable damage to the structure of monolayer assemblies. The six electrophilic self-assembled monolayers were prepared and characterized, and evaluated as substrates for nucleophilic displacement reaction. The intrinsic electrophilicity of the SAMs, and varying nucleophilicity of the incoming chemical species could be utilized to further control surface modification.
Figure 2-17. Reaction kinetics of functionalized organic surfaces monitored by ESCA. Time course for attachment of p-nitrothiphenol to six SAMs bearing iodoacetyl (○), iodobenzyl (●), bromobenzyl (△), chloroacetyl (▲), iodoalkyl (□), and bromoalkyl (■) functionality. Bottom panel shows sulfur signal and the upper panel the nitrogen signal. Data are the mean of two experiments. The Y axis is offset and only shows relative intensity.
Chapter 3. HIV Antigen Attachment for Immunosensor Development Based on Electrophilic Self-Assembled Monolayers

3-1. Introduction

For many years it has been widely recognized that the specific recognition properties of proteins, primarily enzymes and antibodies, can be used to construct electronic or optical biosensors suitable for medical and industrial applications. Figure 3-1 depicts an electrical sensor based on a monolayer bound sensing molecule.

![Diagram](image)

**Figure 3-1.** Organic sensing surface designed for immunosensor. The sensing system consists of solid substrate (oxide layer), subsequent SAM layer (vertical lines upon it), and antigen (Ag) immobilized onto SAM surface (small circle). Antibody (Ab) attachment (big circle) is through specific antigen-antibody binding (d: thickness, SAM: self-assembled monolayer, Ab: antibody, Ag: antigen).
However, very few biosensors\(^1\) are available for practical use due, in part, to the unavailability of peptide attachment methodologies. For a biosensor to be practical, the specific recognition between the two complementary components (sensor & analyte) must be reproducible. It is also important for the biosensor components to be stable toward the analytical environment and for the biological molecules to be strongly bound to the sensor surface. Above all, it is essential that the sensing molecule retain its immunological activity after attachment. Many of the biosensors that have been developed in recent years utilize polymer trapping\(^2\) or membrane barriers\(^3\) to hold the proteins near the sensor surface, making them susceptible to loss of activity through a variety of processes.\(^4\) Hence, developing methods for chemical modification of a surface for biomolecule attachment\(^5\) and / or modification of the protein or peptide\(^6\) prior to attachment is useful to biosensor fabrication.

Non-specific adsorption of proteins and peptides to the surface may lead to many serious problems in practical biosensor development. There have been a number of kinetic and thermodynamic studies\(^7\) on non-specific adsorptions\(^8\) of proteins onto variously modified hydrophilic and hydrophobic solid surfaces. It has been found that methyl-terminated long chain monolayers provide for strong protein physisorption\(^9\) through hydrophobic interactions, whereas hydrophilic hydroxyl and sulfonate-bearing surfaces tend to reduce the non-specific adsorption of proteins.\(^10\) Moreover, it has been demonstrated that PEO (polyethylene oxide) surfaces exhibit little protein adsorption,\(^11\) and that PEO terminated thiolate monolayer assemblies can serve as effective models for studying ways to minimize protein adsorption.\(^12\)

Peptides bearing SH-functionality have been known to strongly chemisorb on gold surfaces.\(^13\) However, non-specific adsorption of the proteins and peptides also occurs, and it allows for their multi-point attachment to the surface.\(^14\) This can result
in a poorly reproducible and very non-uniform layer of adsorbed peptide whose mode of binding can not be easily distinguished. This may be particularly important since there is a possibility that the proteins and peptides can be physisorbed and subsequently deformed by steric repulsion and hydrophobic interaction. Due to the extensive hydrogen bonding of peptides in an aqueous environment, and role of these hydrogen bonds in peptide conformation, this deformation might prevent an antigen layer from being in an active conformation. Such deformation could also lead to cross-linking of the peptides themselves.

The chemistry of peptide or protein attachment which has been developed in recent years has resulted in several methods appropriate for biosensor fabrications. The organic solid surface, among those methods, has emerged as an efficient tool to control protein attachment through covalent bonding. This approach attempts to eliminate problems in protein immobilization such as instability, diffusion, aggregation or inactivation of proteins. For example, a thiol-terminated siloxane-based monolayer derived from either a thioacetate monolayer assembly or disulfide monolayer assemblies was employed for cytochrome c immobilization through a cysteinyl SH group by the creation of a disulfide linkage (R-S-S-R). Carboxylate-terminated thiolate monolayer was used for cytochrome c and cytochrome b₅ attachment by DCC coupling. A bifunctional linker (N-succinimidyl 6-maleimidocaproate, EMCS) was employed to attach the SH-selective maleimide-functional group onto an NH₂-based SAM monolayer for cytochrome c attachment. A short chain NH₂-based SAM, most commonly used for protein attachment, could be modified with glutaraldehyde in water and then reacted with desired proteins or peptides. These approaches provide for robust chemical binding of peptides and proteins to a biosensor surface due to their strong bonding interaction (ionic and covalent bondings), when compared to
physisorption\textsuperscript{23} of peptides or proteins. However, most of these chemical approaches generate irregular surfaces with partially or totally inactivated protein molecules presented in a variety of orientations.

Langmuir-Blodgett films have been used in biosensor design and fabrication. Several L-B films\textsuperscript{24} were successfully employed as efficient biosensors with novel sensing mechanisms. For example, the influenza virus was successfully detected utilizing a colorimetric technique in a biosensor system based on a Langmuir-Blodgett film. However, the need to functionalize the sensing surface with a suitably reactive organic moiety limits the usefulness of L-B techniques in biosensor development.

Self-assembled organic monolayers have shown promise among the substrates used for peptide immobilization, mainly because they remain robust but flexible and can be easily derivatized with functional groups. Simple $S_N2$ reaction chemistry has emerged as an efficient vehicle for peptide attachment. It provides well-defined structures and an uniform array of specific attachment sites. A short chain hydrophobic SAM, (3-iodopropyl)methoxysilane\textsuperscript{25} was used for the covalent attachment of genetically-engineered cytochrome b$_5$. A more reactive chlorobenzyl-containing short-chain siloxane-based monolayer\textsuperscript{26} and a cyanuryl-modified SAM\textsuperscript{27} were employed for urease immobilization through $S_N2$ reaction.

As was discussed in Chapter 2, we have developed six long chain-electrophilic SAMs which allow for efficient surface modification by chemical means. The l-acetyl SAM and BzI SAM, the most reactive among them, are promising candidates for peptide attachment because of their superior reactivity toward relatively unreactive nucleophiles like a neutral thiol.

The focus of the work described in this chapter is primarily on HIV antigen attachment to sensing surfaces for biosensor development (Figure 3-2). A synthetic
peptide was employed as the target antigen to be attached to the SAM surface for
detecting its antibody. The peptide (a fragment of the surface glycoprotein gp-41 on
the HIV virus) has been shown\textsuperscript{28} to react specifically with HIV antibodies. In the
studies reported herein, the most reactive I-acetyl SAM was employed as the reactive
surface film for peptide anchoring. The major goal was to successfully attach the
synthetic HIV antigen peptide to the I-acetyl SAM through its cysteiny1 sulfhydryl
group and to verify that the attached antigen retains its immunological activity (Figure
3-3).

\begin{center}
\begin{tikzpicture}
\node[anchor=east] at (0,0) {Si Substrate};
\node[above] at (0,1) {Ab};
\node[above] at (0,2) {HIV Ag};
\end{tikzpicture}
\end{center}

\textbf{Figure 3-2. HIV antigen for antibody binding.} HIV antigen (HIV Ag) attached to
organic SAM surface senses directly its antibody (Ab) on the Si substrate.
The attachment of peptides and proteins through amino or carboxyl group can often lead to random linkages through many different attachment points, because proteins are rich in such functionality. Since typical cross-linking reagents cannot distinguish a surface amino group from one on another protein molecule, cross-linking and oligomerization of the protein molecules may also occur. In this regard, the Cys residue, bearing a nucleophilic thiol group, offers the possibility for more controlled chemical attachment to the surfaces. Since the thiol group occurs significantly less often than the residues which contain amino (Lys) and carboxyl (Asp, Glu) groups, this should improve chances for oriented attachment. In fact, some proteins (e.g. the FAB' fragment of IgGs) contain only a single free thiol. Thus, the creation of more stable surfaces which react with less abundant side chains like the cysteine residue is of great interest.
To that end, the following experiments, including work with both the antigenic peptides and with several model systems, are described: (a) A tripeptide glutathione was chosen as a model peptide for attachment to the I-acetyl SAM. The specific attachment of this small biomolecule in organic solvent (DMF) was studied with the goal of differentiating between physisorption and covalent attachment. The attachment kinetics of glutathione was measured by a series of ESCA experiments. The selectivity for attachment to the surface was studied with glutathiones containing either free or blocked thiols. (b) A commercially available laminin fragment was employed as a medium-sized peptide for the attachment study. As with glutathione, the attachment of laminin fragment was examined in terms of specificity and selectivity. (c) To model the selectivity and preference for attachment of peptide, the relative reactivity of model compounds with NH₂ or SH within the monolayer environment (DMF, RT, I-acetyl SAM) were qualitatively assessed by obtaining the ratios of ESCA signal intensity from competition studies, which could confirm the preference for reaction with different functionality. (d) HIV antigen, gp-41 synthetic peptide, was attached to the I-acetyl SAM in DMF at RT and the selectivity of its attachment was studied using the same procedures used for glutathione and laminin fragment. (e) An additional peptide based antigen-antibody system, EC-2 peptide in DMF and in water, was employed for comparison with HIV peptide attachment. (f) The antigenic activity of immobilized HIV antigen peptide was studied with the use of ELISA and immunogold labeling techniques. The conclusions of this study include a discussion of the thiol-mediated controlled attachment of HIV synthetic peptide for biosensor development.
3-2. Experimental Section

The syntheses and characterization of the precursors, their SAM formation, ESCA and contact angle measurements, were described in the previous chapter. Glutathione and laminin fragment (residues 925-933) were purchased from Sigma Chemical Co..

3-2-1. Attachment of tripeptide (glutathione) and nonapeptide (laminin fragment) to I-acetyl SAM glass slides

Solutions of glutathione (0.01 M) and of laminin fragment (0.01 M) were prepared in dried DMF at RT. The peptide solutions were used immediately after preparation. Derivatization with glutathione or with laminin fragment was done under dry nitrogen in a glove bag using freshly distilled DMF as the solvent. Freshly derivatized SAM-bearing glass slides were placed in oven dried polyethylene 3 dL vials. For maximum coverage, the I-acetyl SAM slides were immersed in laminin fragment or glutathione solutions at RT overnight. The slides were then rinsed five times on each side with 1 mL portions of DMF followed by similar rinsing with methylene chloride. The peptide-treated SAM slides were dried at RT in air, and stored in a desiccator prior to surface analysis. The contact angle for each slide was measured, and ESCA data for each slide was obtained.

3-2-2. Glutathione attachment kinetics

Eight slides modified with I-acetyl SAM were reacted with 0.01 M solution of glutathione for 0.5 h, 1.0 h, 2.0 h, 3.0 h, 4.0 h, 5 h. Each sample slide was withdrawn from the solution at a given time, and rinsed extensively with DMF, methylene chloride, and acetone. After reaction, these slides were characterized by ESCA.
3-2-3. Blocking of thiol (SH) group of glutathione & laminin fragment

3-2-3-1. Thiol-blocking experiments

Prior to reacting with the I-acetyl SAM, glutathione was treated with a 20 fold excess of dinitrophenyl maleimide in DMF at RT for at least 6 h. The I-acetyl SAM slide was treated with the modified glutathione overnight. Another I-acetyl SAM slide was treated with only the maleimide solution overnight as a control experiment. The cysteinyI thiol group of glutathione was also oxidized by allowing the glutathione solution (in DMF) to remain exposed to air for one week. The resulting solution was then reacted with a freshly derivatized I-acetyl SAM slide overnight. The reactivity of both the maleimide-blocked and oxidized versions was then assessed. In all cases, ESCA was used for monitoring attachment of blocked versus unblocked peptides.

3-2-3-2. Color test for determination of SH group

To ascertain the presence of free thiol in glutathione and laminin fragment, 200 mL of a 0.1 mM solution of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in 0.1 M Tris, pH = 8 was mixed with an equal volume of peptide solution. Adsorptions at 430 nm were recorded. For HIV peptide, a DTNB solution (0.01 M) was prepared in DMF solvent at RT. The availability of free thiol was assessed visually by the presence or absence of yellow color, when the sample was mixed with the DTNB solution.

3-2-3-3. I-acetyl SAM modified optical fiber

A xenon light source was used in conjunction with a scanning monochromator to produce evanescent wave excited fluorescence in the optical probes. This optical source was fed directly into the input fiber of a 2 x 1 fused coupler. The cleaved end of a 200 μ core SiO₂ sensing fiber coated with I-acetyl SAM was connected to the
coupler's output fiber via a micropositioner, which permitted rapid exchange of sensing fibers. The conditions used for SAM coating were similar to those described in chapter 2. The input source light then propagated into the sensing fiber which produced evanescent wave excited fluorescence in the treated section of fiber. This fluorescent emission propagated back through the 2 x 1 coupler and through a narrow band optical filter with the pass band centered in the vicinity of the peak of the emission spectrum. The filtered fluorescent emission was then fed directly to a computer controlled photomultiplier. In this case the excitation spectrum was centered at 350 nm and the fluorescent emission spectrum at 550 nm.

3-2-4. Relative reactivity of NH₂ group vs SH group toward I-acetyl SAM surface

The glass slides, freshly derivatized with I-acetyl SAM, were reacted with 0.01 M 1-decanethiol, or 0.01 M 1-decyl amine, or an equimolar mixture of 1-decanethiol (5 mM) and 1-decyl amine (5 mM). After performing the reactions in DMF at RT overnight, the SAM slides were withdrawn from the solution, rinsed extensively with DMF, CH₂Cl₂, and acetone extensively. The slides were dried at RT in air, and the wetting properties and surface composition were characterized by contact angle and ESCA.

3-2-5. HIV peptide (gp-41 synthetic peptide) attachment to the I-acetyl SAM and Bzl SAM surfaces

I-acetyl SAM and Bzl SAM glass slides were prepared according to the procedure described in chapter 2. XPS, contact angle measurements, and AFM were employed for surface analysis.
3-2-5-1. Attachment of peptides to glass substrates

Solutions of HIV peptide (1.3 x 10^{-5} M) and EC-2 peptide (2.4 x 10^{-5} M) were made under dry nitrogen in a glove bag, using freshly distilled DMF as solvent. Freshly derivatized glass slides were placed in individual oven-dried polyethylene 3-dm vials. Maintaining the inert atmosphere, a 1 mL portion of the respective peptide solution was transferred to each vial. After 12 h, the slides were removed from the vials and rinsed several times with DMF, dried and stored in a dessicator.

3-2-5-2. AFM experiment of peptide-modified SAM surface\textsuperscript{31}

Images of I-acetyl SAM and HIV peptide-treated SAM surfaces were obtained using an atomic force microscope operating in an aqueous environment. Samples were analyzed using a Nanoscope III Atomic Force Microscope (AFM, Digital Instruments) equipped with a fluid cell attachment. I-acetyl SAM glass slides and HIV peptide treated SAM surfaces were placed into a fluid cell attachment for the AFM, and the cell was subsequently filled with 5 mL of phosphate buffered saline (PBS). An initial background scan was made using a 100 μm long, thick-legged triangular Si₃N₄ cantilever with an integrated pyramidal tip. Typical engagement forces were in the range of 5-10 nN for both images.

3-2-5-3. Ellipsometric measurements\textsuperscript{32}

The ellipsometric thickness measurements were carried out with a variable angle spectroscopic ellipsometer from J. A. Woollam Company (polarizer-sample-rotating analyzer configuration). The xenon lamp was used as a light source with a 1 mm spot size set by means of a detector aperture. The ellipsometric spectra were acquired from the area of a thin silicon dioxide within the window at angles of
incidence from 66° through 71° at a 1° increment and at wavelengths from 300 to 400 nm at a 10 nm increment. Prior to acquisition of data, the instrument was calibrated with the sample of 250 Å thick silicon dioxide on silicon wafer. Each data point was averaged over 30 revolutions of the analyzer. Computer-driven stepper motors and a position sensitive quadrant silicon detector allowed the incident angles to be adjusted with a precision of 0.005°.

3-2-5-4. Blocking experiments of HIV peptide with N-ethyl maleimide (NEM)

Prior to reacting with the I-acetyl SAM, HIV peptide was treated with a 20 fold excess of N-ethyl maleimide (NEM) in DMF at RT for at least 2 hrs. An I-acetyl SAM slide was then treated with this modified HIV peptide overnight. Another I-acetyl SAM slide was treated with only the maleimide solution overnight as a control experiment. In all cases, ESCA was used for monitoring attachment of blocked or unblocked peptides. To verify completeness of the peptide blocking, 50 mL of a 0.1 mM solution of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in DMF was mixed with an equal volume of modified peptide solution. The DTNB solution (0.01 M) was prepared in DMF solvent at RT. The availability of free thiol was assessed visually when the sample was mixed with the DTNB solution by the presence or absence of yellow color. The presence of free thiol was reflected from the observation of an yellow color.

3-2-5-5. ELISA on the HIV peptide derivatized organic substrates

The antigenicity of both HIV peptide and EC-2 peptide were confirmed by Enzyme-Linked Immunoabsorbant Assay (ELISA) performed on both the free peptides and on the peptides once they were attached to α-iodoacetyl SAM.
For iodoacetyl SAM coated with HIV antigen, ELISA was performed as follows. The 24 wells containing coverslips bearing I-acetyl SAMs (treated with HIV synthetic peptide) were rinsed with PBS wash (a solution of bovine serum albumin, tween-20 and PBS solution), prior to addition of antiserum (diluted 100:1 in PBS wash) to half of the wells and pre-immune serum (diluted 100:1) to the other half. I-acetyl SAM slides treated with HIV peptide were placed into the wells. After a 2 hour incubation, the glass slides in the wells were washed with PBS solution, and the horseradish peroxidase conjugate (diluted 10,000:1) was added to all the wells. After a final rinsing with PBS solution, the SAM glass slides were developed with 2,2'-azino-bis(ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABPS) solution. The absorbence was measured at 415 nm.

3-2-5-6. Immunogold labeling experiments

For scanning electron microscopic analysis of these surfaces, a previously described method for immunogold labelling with silver enhanced gold beads was used and performed. The I-acetyl SAM slides, treated with HIV peptide, were rinsed in phosphate buffered saline (PBS). First, non-specific protein binding sites were blocked with 15 % nonfat dry milk in PBS with 2 mM Na$_2$EDTA. Unlabeled protein A (0.2 mg/mL) from *S. aureus*, Cowan strain (Sigma Chemicals) was added to this mixture to block Fc portions of adsorbed plasma immunoglobulins and the samples were incubated for 1 h at 37 °C. The slides were rinsed in PBS and then incubated with primary rabbit antisera or control nonimmune sera (1:10 to 1:100 dilutions in PBS used) for 1 hour at 37 °C. The samples were rinsed in PBS and fixed in 2.5 % glutaraldehyde (Grade 1, Sigma Chemicals) overnight at 4 °C. Subsequently, the slides were again rinsed in PBS and any remaining glutaraldehyde was neutralized with 50
mM glycine for 20 min. Protein A-gold complex (10nm gold beads, AuroProbe-G10, Amersham Life Sciences) was diluted 1:75 with PBS and incubated with the samples for 1 hour at 37 °C; the samples were rinsed with PBS and the gold beads enhanced to 100-200 nm using a commercial silver enhancement kit (IntenSe BL, Amersham). All slides were rinsed in distilled water, followed by 30%, 70%, 90%, and 100% alcohol. Samples were further dehydrated using hexadimethylsiloxane, vacuum dried and then processed for scanning electron microscopy by sputter coating of the samples with gold/palladium. The samples were then processed for routine scanning electron microscopy using a JEOL Model 840A (JEOL, Tokyo, Japan) at a working distance of approximately 22 mm and a 25kV accelerating voltage. Photomicrographs were taken at various magnifications.

3-3. Results and Discussion

Simple, short chain SAMs of aminosilanes (APS)\textsuperscript{35} have been extensively used as a basis for linkage of protein to the amino-rich surface by cross-linking through the pendant amino or carboxyl side chains of the protein. However, the polar, short chain \textit{NH}_2-bearing film is susceptible to hydrolysis despite possible improvements of the deposition procedures\textsuperscript{20} and the protein layers made using this method have been demonstrated to be unstable and highly disordered.\textsuperscript{36}

Unlike short chain \textit{NH}_2-SAMs, long chain electrophilic self-assembled monolayers are robust enough to survive the hydrolytic environment. The hydrophobic alkyl chains of such films serve as a barrier to prevent hydrolysis chemistry from occurring within the underlying siloxane linkages. Hence, long chain films bearing electrophilic functional groups were designed to provide a highly ordered, stable, vehicle for peptide attachment to surfaces.
3-3-1. A model study of specificity: glutathione and laminin fragment

3-3-1-1. Attachment of glutathione to I-acetyl SAM surface

A study of selective peptide attachment was conducted with the tripeptide glutathione, which contains two carboxyl groups, an amino group, and a thiol group (Figure 3-4).

\[
\text{HO}_2\text{C-CH-CH}_2\text{-CH}_2\text{-C-NH-CH-C-NH-CH}_2\text{O} \quad \text{O} \quad \text{CO}_2\text{H}
\]

\[
\text{NH}_2 \quad \text{CH}_2 \quad \text{SH}
\]

**Figure 3-4. Structure of glutathione: γ-Glu-Cys-Gly.**

This peptide was found to react with SAM modified surfaces in a way that was comparable to that of the model thiols described in chapter 2. Attachment was demonstrated by the parallel appearance of ESCA signals for carbonyl carbon, sulfur, and nitrogen signals, and the disappearance of iodide signal (Figure 3-5). The strong appearance of N and S signals of the glutathione-treated SAM glass slides were in contrast with the disappearance of I peak after the treatment. These results nicely parallel those obtained for the reaction of I-acetyl SAM with p-nitrothiophenol (PNTP). Moreover, whereas PNTP attachment to various SAMs left the surface wetting properties minimally changed (advancing water contact angles of 75-78°; receding water contact angles of 70-75°), the attachment of glutathione, with all of its polar functionality, created hydrophilic surfaces with advancing contact angles below 30°.
Figure 3-5. Glutathione attachment to I-acetyl SAM surface. XPS signals of C peak (a), I peak (b), S peak (c), and N peak (d) of glutathione attached SAM surface. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.

The reaction of glutathione with I-acetyl SAM surfaces was monitored by ESCA as a function of time (Figure 3-6). The data obtained from ESCA is displayed by plotting both the S and N signals versus time. Figure 3-6 shows the time course for the reaction of glutathione with an α-iodoacetyl SAM surface. While there is some scatter in this data, presumably caused by the ex situ monitoring of each set of SAM glass slides, the parallel behavior of the two different probe signals makes a convincing case for the expected course of peptide attachment.
Figure 3-6. Glutathione attachment kinetics of I-acetyl SAM glass slides. The kinetics of glutathione attachment was indicated by increase in N (nitrogen) and S (sulfur) XPS signals. The Y axis is relative signal intensity in counts per electronvolts.

3-3-1-2. Attachment studies of thiol-blocked glutathione: selectivity

The thiol side chains of Cys residues provide an alternative to amino or carboxyl groups as points of attachment. Cys is less frequently found, and, in proteins, its thiol is often involved in disulfide bonds which further reduce the availability of thiol nucleophiles. In synthetic peptides known to represent epitopes of specific antigens, it is less common to find Cys residues than amino or carboxyl groups. Moreover, addition of a Cys residue to a synthetic antigen may well render it reactive with surfaces such as those we describe, while not interfering with biological interactions important for its function.
Since glutathione contains nucleophilic residues (amino and carboxyl) other than the thiol side chain of the Cys, further examination of the role of the Cys residue in peptide attachment was undertaken (Figure 3-7). The peptide was modified with a maleimide derivative (dinitrophenylmaleimide, DNPM), commonly used to block thiols in proteins. The disappearance of free thiol was established by using Ellman's reagent.\textsuperscript{37} Freshly derivatized I-acetyl SAM glass slides were treated with the modified glutathione solution (SH-blocked glutathione with maleimide). The SAM glass slides, withdrawn from the derivatized glutathione solution, were rinsed with DMF extensively, and characterized by ESCA. As was shown in Figure 3-7, treatment of the α-iodoacetyl SAM surface with the DNPM-blocked glutathione, generate neither a nitrogen signal nor a sulfur signal on the organic surface, suggesting that a free thiol group is required for peptide attachment. This is consistent with the blocked glutathione-treated SAM surface showing no appreciable change in wetting property (advancing water contact angle: 66° ± 3°).

Although DNPM modification is specific for thiols in aqueous solution (under conditions where amino groups are protonated), it was possible that, in DMF, DNPM might also have blocked peptide amino groups. Therefore, the reactivity of air-oxidized peptide was also examined (Figure 3-8). Glutathione solution oxidized in the air was employed in this blocking experiment for treatment of I-acetyl SAM glass slides. As was shown in Figure 3-8, using air-oxidized glutathione which contained disulfide and/or sulfonic acid instead of a free thiol group, peptide attachment to the α-iodoacetyl SAM surfaces could not be observed. Since air oxidation of amines is very unlikely under these conditions, this result confirms the thiol residue as the attachment site of peptide.
Figure 3-7. 1-acetyl SAM treated with maleimide-treated glutathione. Sulfur (top) and nitrogen (bottom) XPS signals for iodoacetyl SAM treated with glutathione (1) and with maleimide-treated Glutathione (2). The Y axis is offset and only shows relative intensity.
Figure 3-8. I-Acetyl SAM treated with air-oxidized glutathione. Sulfur (top) and nitrogen (bottom) XPS signals of iodoacetyl SAMs treated with glutathione and air-oxidized glutathione. Oxidation of the peptide thiol was confirmed by test for free thiol with Ellman's reagent. The Y axis is offset and only shows relative intensity.
The presence of free amino groups in covalently-attached glutathione on the SAM surface can be detected with further modification by either dansyl chloride or rhodamine B isothiocyanate. Following either of these secondary modifications, the presence of sulfur is seen by ESCA, indicating the attachment of the reagent to the peptide amino group. This would not be the case if peptide attachment to the I-acetyl SAM were through its amino groups.

This selective attachment of glutathione to I-acetyl SAM surface was reproduced on a fiber-optic sensing device (Figure 3-9). The optical fiber coated with I-acetyl SAM was immersed into glutathione in DMF as was done to the flat SAM slides. This was followed by the dansyl chloride treatment in methylene chloride.

![Diagram](image-url)

**Figure 3-9.** Fiber-optic detection of the presence of glutathione. (1) Glutathione-treated I-acetyl SAM optical-fiber surface (◼). (2) A fiber surface treated with dansyl chloride (□). (3) The difference between the two samples for comparison (X).
at RT for 4 h. The fluorescence peak observed at 550 nm, based on input excitation peak at 350 nm, confirmed that the free NH₂ functional group of attached glutathione can be further modified with a dansyl moiety. While this result only requires that some, not all, of the glutathione amine groups be available for dansylation; it is consistent with the experiments where glutathione was found to react with the I-acetyl SAM selectively through its cysteiny1 SH group. This model study of glutathione attachment supports the idea that the I-acetyl SAM is useful for selective peptide attachment in organic medium through cysteine residue.

3-3-1-3. Laminin fragment attachment to I-acetyl SAM surface

Additional studies were conducted with a nonapeptide fragment of the protein laminin (Figure 3-10). With the exception of the ε-amino group of Lys, this peptide contains all the nucleophilic functionalities of typical proteins including α-amino, carboxyl, thiol, phenol, and alcohol groups.

**Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂**

*Figure 3-10. Structure of laminin fragment.*

This highly-soluble (DMF) laminin fragment reacted with the I-acetyl SAM under the same conditions used for the glutathione (Figure 3-11). The key diagnostic of peptide attachment is the nitrogen XPS signal. As with glutathione, the laminin fragment strongly attached to the surface and iodide was displaced. While ESCA showed an amidic C signal (288.6 eV), interestingly, with surface-bound laminin fragment we observed no sulfur XPS signal, even with an angle-resolved XPS
Figure 3-11. I-acetyl SAM treated with laminin fragment. XPS signals of carbon region (a), nitrogen region (b), sulfur region (c) and iodide region (d). The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 unit.
experiment. This result is presumably a combination of the inherently small sulfur signal (low XPS sensitivity factor) being further attenuated by the mass of the attached peptide. This is consistent with the sulfur being the attachment site for the peptide and thus putting it in a position where it is most shielded for XPS by the remainder of the nonapeptide molecule.

Further confirmation of peptide attachment was obtained by monitoring surface wetting properties. Since the laminin fragment is, overall, a less polar molecule than glutathione, it was expected that surfaces bearing this peptide would not be as wettable as those bearing glutathione. This was confirmed by observing a water contact angle of 45° (adv.) - 40° (rec.) for surfaces bearing laminin fragment peptide in comparison with an advancing contact angle of <30° for glutathione bearing surfaces.

3-3-1-4. SH-blocked laminin fragment attachment to I-acetyl SAM

Demonstration that this reaction occurred through the thiol side chain instead of an amine group is again indicated by the lack of reaction with the DNPM-blocked laminin fragment peptide (Figure 3-12). With DNPM-blocked laminin peptide, neither iodide displacement was observed, nor nitrogen signal was detected in the ESCA experiment. This indicates that the presence of SH group is essential in peptide attachment.
Figure 3-12. I-acetyl SAM with maleimide-treated laminin fragment. Attachment of laminin fragment to iodoacetyl SAMs: XPS signal for nitrogen (top) obtained from SAMs exposed to native peptide and maleimide blocked peptide; XPS signal for iodide (bottom) observed with native and maleimide blocked nonapeptide. The Y axis is offset and shows only relative intensity.
3-3-1-5. The relative reactivity of alkyl \( \text{NH}_2 \) vs alkane \( \text{SH} \) with I-acetyl SAM

In further exploring the reactivity of our SAM systems (Figure 3-13), it was found that the \( \alpha \)-iodoacetyl SAM surfaces can be made to react with alkyl amines. Thus, the possibility that amines of a protein could react with the SAM electrophilic functional groups was real (not listed in Figure 3-13). Indeed, in the absence of

Figure 3-13. XPS of competition experiments (reactivity of \( \text{NH}_2 \) vs \( \text{SH} \)). XPS signals of I region (a), C region (b), N region (c), and S region (d). I-Acetyl SAM glass slides were treated with equimolar solution of decanethiol and decyl amine in DMF at RT overnight. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
competing thiol reactivity with amines could be observed. However, the thiol functional group was highly preferred in the surface reaction. This was demonstrated by competition experiments with an equimolar mixture of decyl amine and decanethiol in DMF. Under these conditions, the thiol compound was preferentially bound to the surface, indicating that the bonding through thiol (SH) of long chain alkyl olefin compound is preferred.

3-3-2. Selective attachment of HIV antigen to the I-acetyl SAM surface

The experiments reported previously all suggested that peptides and proteins can be immobilized onto the organic SAM surface by covalent binding through a specific functional group. The gp-41 synthetic peptide, a fragment of major glycoprotein in the viral envelope of the HIV virus, was selected for the study as a HIV antigen molecule. EC-2 peptide, a synthetic fragment of the second extracellular loop of a membrane receptor (the parathyroid hormone receptor), was chosen for comparison purposes. In both cases, we planned to use organic solvents (DMF or acetonitrile) for the attachment chemistry to I-acetyl SAM surfaces. The peptide attachment method employed here is essentially the same as that used for attaching glutathione and laminin fragments to glass substrates, through a variety of electrophilic self-assembled monolayers. The SAMs chosen for gp-41 synthetic peptide immobilization, I-acetyl SAM and I-benzyl SAM, were prepared according to the procedure previously described. Well-dried DMF was used as solvent for these reactions. Beyond simply achieving successful thiol-mediated attachment, HIV peptide must retain its antigenic property in order to have applications in immunosensor technology.
3-3-2-1. HIV antigen attachment to I-acetyl SAM

HIV antigen (Figure 3-14), a 20 amino-acid synthetic peptide whose cysteiny1 SH group is positioned near the peptide C-terminus, is water-insoluble but soluble in DMF and acetonitrile. It is also quite hydrophobic when compared to the laminin fragment. The peptide contains a single thiol (Cys), two guanidino groups (Arg), a phenol (Tyr), and a single alcohol group (Ser). The use of DMF for HIV peptide attachment is beneficial both to promote surface reaction and to remove any remaining unbound HIV peptide after the reaction is complete.

Arg-Ile-Leu-Ala-Val-Glu-Arg
Leu-Leu-Gln-Asp-Lys-Leu-Tyr
Gly-Ile-Trp-Gly-Cys-Ser

Figure 3-14. Structure of HIV synthetic peptide.

Benzyl iodide SAMs were demonstrated to also act as a substrate for HIV peptide attachment (Figure 3-15). BzI SAM was prepared from BzBr SAM by halide exchange. The BzI SAM was then treated with 10^-5 M HIV peptide solution for 8 h. The ESCA shows the strong N peak and C peak along with disappearance of I peak, indicating the BzI SAM can serve for peptide attachment. This surface, however, has the disadvantage of being photolytically unstable and hence long-term storage changes its surface composition. The wetting behavior of three month old BzBr SAM (precursor to BzI SAM) suggested that it is decomposed to a SAM with more
hydrophobic surface properties. Specifically, the contact angle went from 88° to 95°.
Thus, most subsequent work was done on the L-acetyl SAM surface.

Figure 3-15. Bzl-SAM treated with HIV synthetic peptide. XPS signals of N region (a) and l region (b) of HIV peptide attached Bzl SAM. XPS signals of Br (c) and C (d) peaks from 3 month old BzBr SAM glass slide. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
Solutions of HIV peptide in DMF ranging from $10^{-4}$ to $10^{-8}$ M were prepared (Figure 3-16). These HIV peptide solutions were used for treatment of I-acetyl SAM glass slides. Based on the N intensities of each SAM slide treated with HIV peptide solutions, Figure 3-16 shows that $10^{-4}$ M is a solution concentration appropriate for attaching HIV peptide to the α-iodoacetyl SAM surface. It clearly shows the substantial surface N signal that is obtained by using HIV peptide at this concentration.

![Graph showing XPS signals of N peaks of HIV peptide treated α-I-acetyl SAM slides at different concentrations (1. 10^{-4} M; 2. 10^{-4} M; 3. 10^{-4} M). The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.]

Figure 3-16. I-acetyl SAM treated with different concentrations of HIV peptide. XPS signals of N peaks of HIV peptide treated α-I-acetyl SAM slides were monitored at different concentrations (1. $10^{-4}$ M; 2. $10^{-4}$ M; 3. $10^{-4}$ M). The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.

The attachment of HIV peptide to I-acetyl SAM is established by the appearance of a strong N signal and the characteristic C peak in the ESCA experiments.
(Figure 3-17). The strong N peak and carbonyl C peak (O=CNH−: 288.76 eV, O=C−O: 287.32 eV, O−C−CH₂−: 285.92 eV and -CH₂−: 284.45 eV) indicate that the I-acetyl SAM surface is modified with HIV synthetic peptide. These signals survived extensive rinsing with DMF, and a continuous stream of methylene chloride in a

Figure 3-17. HIV peptide attachment onto I-acetyl SAM surface. XPS signals of I region (a), N region (b), S region (c), and C region (d), the resolved C peak (e) from I-acetyl SAM slide treated with HIV peptide solution for 12 h. The characteristic carbon peak was resolved into 4 different peaks. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
Soxhlet extractor. The wetting property of the organic SAM surface does not change enough to distinguish the modified organic surface from the I-acetyl SAM, simply due to hydrophobicity of HIV synthetic peptide.

AFM images of I-acetyl SAM and HIV-P modified SAM surfaces were obtained (Figure 3-18). In general, AFM allows direct visualization of images of the peptide-treated SAM surfaces. AFM image of a surface derivatized with HIV peptide reveals clear evidence for peptide attachment, when compared to that of I-acetyl SAM. The I-acetyl SAM surface is seen to be relatively smooth on the 5 nm vertical scale, while the peptide modified surface is not. However, this data does not provide any specific information regarding the quantitative thickness of HIV peptide immobilized on the SAM surface except different surface topography.

![AFM images](image)

Figure 3-18. AFM images of HIV-P modified SAM surface vs I-acetyl SAM surface. (a) shows a 500 nm scan of a derivatized monolayer composed of I-acetyl SAM. (b) represents I-acetyl SAM treated with HIV peptide for 6 h. The topography for both images is apparent with a 5 nm z-scale and at 100 nm scan size.
The reaction of HIV synthetic peptide with I-acetyl SAM surfaces was monitored by ESCA as a function of time. The data obtained from ESCA N intensity was plotted against time. Figure 3-19 shows the time course for the reaction of HIV synthetic peptide with an iodoacetyl SAM surface. Based on such experiments, attachment times of 4-6 h were considered to be adequate for surface maximum coverage with HIV peptide.

Figure 3-19. HIV synthetic peptide attachment kinetics. The attachment of HIV peptide was followed by monitoring the N signal intensity as a function of time. (x-axis: time, y-axis: arbitrary counts of N intensity reaching surface saturation with peptide)
3-3-2-2. Non-specific adsorption study of HIV synthetic peptide

The question of non-specific HIV peptide adsorption was also explored. HIV synthetic peptide initially appears to attach to long chain hydrophobic films of octadecyl trichlorosilane (OTS). This requires that the peptide solution be in direct contact with the SAM surface for 8 h. However, it was found that the peptide is completely removed by repeated rinsing with DMF (Figure 3-20). This data is consistent with physisorption of HIV peptide onto the unfunctionalized OTS SAM surface in DMF at RT, but subsequent peptide desorption. No peptide adsorption occurred with bare glass slides as was the case with OTS SAM. N signal was not detected with HIV peptide-treated bare glass slides.

![Graphs showing adsorption data](image)

Figure 3-20. Bare SiO₂ glass and OTS SAM treated with HIV peptide. N region (a) of Bare SiO₂ glass treated with HIV peptide. N region (b) of unfunctionalized OTS film treated with HIV peptide. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.

HIV synthetic peptide contains nucleophilic sites for attachment to the reactive SAM surfaces. Therefore a blocking experiment, as a negative control, was performed in order to demonstrate that HIV peptide is being immobilized by selective covalent
binding through the sulphydryl group of its Cys residue. The HIV peptide was treated with N-ethylmaleimide (NEM) in DMF to block the Cys thiol group. This blocking was monitored with DTNB in DMF. Treatment of I-acetyl SAM with blocked HIV peptide solution for 8 h showed (by ESCA) no HIV peptide on the I-acetyl SAM (no N, carboxyl C peaks) (Figure 3-21). This clearly indicates that HIV peptide attachment to I-acetyl SAM surface under normal conditions is selective for attachment via the SH group. As had been observed for the smaller peptides (glutathione and laminin fragment), once the cysteine thiol is blocked, no HIV peptide attachment is observed. Moreover, not only is no N ESCA signal seen, but the iodide signal persists.

Figure 3-21. I-acetyl SAM treated with SH-blocked HIV peptide. XPS signals of N (a), I (b) and C (c) peaks indicate that the attachment require the presence of cysteinyi SH group. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
This requirement of electrophilically reactive functionality for HIV peptide attachment was verified by a SAM surface-blocking experiment prior to peptide solution treatment. In order to block the attachment site of the SAM, an I-acetyl SAM was first treated with either \( \rho \)-nitrothiophenol or decanethiol for 8 h at RT. This treated surface was then immersed in a solution of HIV peptide for 8 h. The glass slide, withdrawn from the peptide solution, was rinsed and characterized by ESCA (Figure 3-22). In this experiment, while the I ESCA signal does disappear, no HIV peptide can be detected on this attachment-site blocked surface. This provided further confirmation that when the peptide does attach to the surface, it is by specific and selective covalent binding through its Cys SH group.

![Graphs](image)

**Figure 3-22.** I-acetyl blocked SAM treated with HIV peptide. XPS signals of C (a), N (b), I (c), and S (d) regions of decanethiol-modified I-acetyl SAM. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 unit.
The thickness of the HIV peptide modified SAM surface was measured by ellipsometry, to be, on average, 6-7 Å.\textsuperscript{32,40} The complete displacement of iodide by the thiol of the HIV peptide is unlikely due to the structural bulkiness of the biomolecule immobilized on the surface. This ellipsometric data (Figure 3-23)\textsuperscript{32} is consistent with a geometry in which the peptide is extended horizontally along the surface. It suggests that other displacement processes (perhaps with adventitious water) may be responsible for the total disappearance of I seen in Figure 3-17.

![Thickness measurement of HIV modified SAM surface. The thickness of HIV peptide attachment was monitored by ellipsometry as a function of time.](image)

3-3-2-3. HIV synthetic peptide vs EC-2 peptide in organic and aqueous solvents

As a comparison to HIV synthetic peptide attachment, EC-2 peptide (Figure 3-24), an extracellular segment of the membrane receptor for thyrotoxic hormone, was
immobilized on an I-acetyl SAM using the same strategy (RT, DMF, 10^{-4} \text{ M}, 8 \text{ h}). The amount of EC-2 immobilized on the surface was much smaller (1.68 \% normalized against C peak), based on N peak intensities, than the amount (4.57 \% normalized against C peak) of HIV peptide attached under the same condition. The thickness of EC-2 immobilized on the SAM was estimated to be less than that of HIV peptide immobilized surface. (2-3 \text{ Å} compared to 6-7 \text{ Å} for HIV peptide). This could be attributed to the accessibility of the reactive SH group in HIV peptide by virtue of

![Chemical Structure](image)

**Figure 3-24.** I-Acetyl SAM treated with EC-2 peptide in DMF. ESCA signals of C (a) and N (b) regions of EC-2 attached SAM. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
its placement near the end of the peptide chain. In the case of EC-2 peptide, lower solubility in DMF and having its Cys SH group buried in the middle of the molecular chain of the peptide may adversely affect the nucleophilic substitution of the I-acetyl SAM. In all cases, the unreacted EC-2 peptide could be removed with simple DMF rinsing.

An interesting set of observations emerged when EC-2 adsorption was done from water instead of from DMF (Figure 3-25). EC-2 peptide, dissolved in water, absorbed onto a variety of hydrophilic surfaces, including bare Si wafer and a diverse array of functionalized SAMs, including the hydrophobic OTS SAM. The strong adsorption of EC-2 peptide to OTS SAM in aqueous medium is in sharp contrast to the lack of detectable adsorption of EC-2, in DMF.

![Graph](image)

**Figure 3-25.** OTS SAM treated with EC-2 in water. XPS signal of N peak. The strong N peak demonstrates the EC-2 attachment, indicating that it is physisorbed onto OTS SAM. The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
Water could also be employed as solvent in anchoring EC-2 peptide to the I-acetyl SAM slides containing the reactive site for attachment (Figure 3-26). A significant amount of existing I peak after the treatment suggests that EC-2 peptide was attached to the functionalized I-acetyl SAM by non-specific binding, whose amount was significant when compared to that obtained from DMF. The ratio of N peak against the C peak (14.83 %) of EC-2 peptide attached from water was almost 7 times larger than that (1.68 %) of EC-2 peptide attached from DMF solvent. These results indicate the EC-2 peptide in water adsorbs onto the organic SAM surfaces very differently than from DMF solution.

As was shown above, DMF as solvent has the advantage of minimizing hydrolysis of the very reactive α-iodoacetyl SAM surface. To use organic solvent in peptide attachment is advantageous in terms of faster kinetics and better hydrolytic stability of the surface, provided that the given biomolecule could be dissolved and could retain its biological activity in the solvent. This shift to organic solvent parallels the work of Klibanov et al., who found that organic solvents (e.g. acetone, DMF) can be used to advantage in chemistry of enzymatic organic reactions. In our work, this strategy was effective for HIV peptide but not for EC-2 peptide. This highlights the need for each new peptide attachment problem to be independently analyzed.
Figure 3-26. I-acetyl SAM treated with EC-2 peptide in water solvent. XPS signal of S (a), N (b), I (c), C (d) regions of EC-2 treated I-acetyl SAM. The survey scan (e) showed the relative intensity of N vs C peaks (14.83 % N intensity ratioed against C peak). The Y axis is relative signal intensity with the largest signal (peak or noise) normalized to a value of 10 units.
3-3-3. Antigenicity of HIV peptide immobilized on the surface: ELISA and immunogold labelling method.

The antigenicity of HIV peptide immobilized onto the 1-acetyl SAM surface was studied using ELISA,\textsuperscript{33} as a preliminary study, and then immunogold labelling experiments\textsuperscript{34} for the HIV-modified SAM glass slides.

The peptide coated SAM surfaces were exposed to immune-sera and pre-immune sera (as a control) from rabbits. ELISA was performed for 1-acetyl SAM slides derivatized with HIV peptide. HIV peptide immobilized SAM slides were

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3-27}
\caption{ELISA of HIV-immobilized SAM slides. Antigenicity of 1-acetyl SAM slides derivatized with HIV peptide at different concentrations were assessed by ELISA. Primary antibody dilution of 1:100 was used.}
\end{figure}
treated with the antiserum which were raised against gp-41 synthetic peptide for enzyme-linked immunosorbent assay (ELISA). The SAM glass slides thus prepared were treated with a secondary antibody labeled with enzyme (horseradish peroxidase). The absorbance was monitored for the slides treated with ABPS solution in an automatic reader. The variations at different concentrations were noted in order to adjust the concentrations of HIV peptide to those appropriate for meaningful operation (Figure 3-27).

Silver-enhanced immunogold labeling experiments were also used to visualize the presence of HIV antigen on the SAM surface. The N-acetyl SAM glass slides treated with HIV antigen were demonstrated to bind protein A gold complex, which was visualized by SEM. SEM pictures of HIV-antigen coated SAM glass slides indicate HIV antigen bound to the SAM surface consistent with the HIV antigen having retained its antigenic activity. This was confirmed by a negative control experiment, by glass slides prepared from both pre-immune sera and non-reactive species on the surface itself. It was found that no particles were seen with surfaces exposed to a preimmune serum, and the number of particles was a function of exposure time to antibody and the fractional saturation of the surface with antigen. The antibody coverage is not uniform (Figure 3-28).

These results confirm that HIV antigen does attach to the surfaces and appears to reach a maximum coverage. Much of the antigen is attached through the thiol side chain (cysteine residue), and at least a fraction of the bound antigen will bind antibodies. The immunologically active antibody molecules on the surface are not closely packed, and may be rather widely distributed. No definite interpretation of the different size particles seen in the immunological assay is available. Coverage of the surface with antibody does not appear to approach saturation. Using the SAM to
generate uniform and stable surfaces which are reactive against a specific single site in antigen peptides, we have been able to retain biological activity in at least a portion of the attached peptides.

Figure 3-28. Immunogold labelling SEM for HIV antigen treated with its antibody. Distribution of antibody molecules on α-iodoacetyl SAM-coated glass surfaces treated with HIV peptide. Particles represent gold beads of 50-150 nm diameter (10 nm gold beads) attached to second antibody by protein (a) & (b): HIV antigen-treated surfaces exposed to HIV antibody: In (a) antigen loading proceeded for 15 min, in (b) antigen loading was for 6 h. Antibody-treated surfaces not treated with HIV antigen (d) and HIV modified SAM surface exposed to non-immune sera (c).
3-4. Concluding remark

In many applications requiring biologically active proteins attached to inorganic surfaces, it is important to have surfaces which are structurally well-defined and still versatile enough to attach different proteins or peptides. For immunosensors, one can envision surface-bound antigen peptides attached to a sensor capable of detecting a specific antibody. Such peptides can be of different compositions and may require surfaces with different chemical and / or physical properties. Although the amino and carboxyl side chains are the most likely candidates for chemical attachment sites and the most often used, these side chains are also likely to be involved in the biological activity of the peptide or protein, and they are the least likely to provide a unique site for attachment. Thus, it is important to develop surfaces based on a range of attachment methods to accommodate various applications.

Our studies with the set of electrophilic SAMs described above, indicate that they are quite specific for the thiol side chain in peptides. It must be concluded, however, that since this reaction selectivity is not absolute, some anchoring of peptides to our electrophilic SAMs in the event of lysine-rich peptide (untested) is possible. Specifically, the α-amino group in the peptides we have used is less nucleophilic than the ε-amino group in the common amino acid, lysine. Thus, we can not rule out the possibility that a peptide rich in lysine functionality might react to some extent through this residue.

The most reactive SAM surface, t-acetyl, is proposed as the basis for biosensor fabrication. SAM reactivity with cysteine-containing peptides was demonstrated. Blocking experiments demonstrated the selectivity of peptide attachment through thiol functionality. Given that the peptides tested contain all the
nucleophilic side chains found in proteins (thiol, alcohol, phenol, carboxyl, and amine),
the selective blocking experiments indicate that these SAMs will be useful for the
directed attachment through cysteine side chains in proteins and peptides. The
flexibility of electrophilic character inherent in our systems is of great advantage in its
ability to control the electrophilic character of the surface and thus should allow
customize surface reactivity to adjust for various circumstances.

We utilized DMF as solvent, to avoid hydrolysis of the α-iodoacetyl surface.
The use of such an organic solvent facilitated the rapid attachment of biomolecules
onto the surface, however, some peptides may not be soluble in non-aqueous systems
or may lose biological activity in such solvents. Using organic solvent can positively
affect the chemistry of peptide attachment. In general, the reaction of organic
monolayer surfaces in organic medium render the peptide attachment favorably easier
and more efficient.

The successful manipulation of the α-iodoacetyl SAM for selective peptide
attachments was demonstrated for the HIV peptide attachment. The selective
attachment of HIV peptide, synthetic gp-41 peptide, through cysteine sulfhydryl group
was achieved in DMF at RT. The antigenicity of an immobilized HIV peptide
monolayer was retained, as confirmed by ELISA and immunogold labelling. This
methodology provides the basis for biosensor fabrication on surfaces amenable to
electrochemical and/or optical detection techniques.
Chapter 1


Chapter 2


Chapter 3


30. The fiber-optics experiments were performed in a collaboration with Professor E. F. Carone group and his group at John-Carroll University.

31. AFM images of SAM-treated surfaces were obtained by Mr. Chris Siedlecki of Professor R. E. Marchant's group in the Biomedical Engineering Department at CWRU.

32. The thickness measurement of peptide-modified organic surface was obtained by Mr. Dmitri Vezenov.

33. The ELISA was performed by Dr. Jerry-Reed Mundell in Chemistry Department at CWRU.

34. The immunogold labelling of HIV-modified SAM glass slides were performed in a collaboration with Dr. N. P. Ziats in the Institute of Pathology at CWRU.


